Effects of neurotrophin-3 (NT-3) administration on gene expression and dorsal root ganglion neuron loss and repair following axotomy in adult rats

by

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ABSTRACT

Following sciatic nerve transection in adult rats, a proportion of injured dorsal root ganglion (DRG) neurons die, through apoptosis, over the following 6 months. Axotomy also has effects on expression of neurotrophins (NGF, BDNF, and NT-3) and their receptors (trkA, trkB, trkC, and $p75^{NTR}$) in DRG as shown by *in situ* hybridisation or Northern blotting. Previous groups showed that administration of neurotrophin-3 (NT-3) to the proximal stump or intrathecally appears to prevent neuronal loss and functional impairment after axotomy.

This thesis tests the hypothesis that

(1) Axotomy may cause certain cells to differentiate into DRG neurons and NT-3 may stimulate this process.

(2) Systemic NT-3 may produce morphological and biochemical changes in DRG that may assist regeneration.

During the course of the study, the 4^{th} and 5^{th} lumbar DRGs were examined up to 8 weeks after right sciatic nerve transection and ligation. Stereology was used to estimate neuronal numbers, and morphological and immunohistochemical techniques were used to examine the incidence of neuronal apoptosis. Antibodies for β-III tubulin, trkA, trkC and CGRP were applied to characterise nestin-immunoreactive cells. Real-time quantitative PCR was used to investigate the effects of axotomy and systemic NT-3 on the mRNA expression of neurotrophins, their receptors and nestin in injured DRGs at various time points. In addition, the effects of axotomy and NT-3 treatment on neuronal genes were investigated by microarray.

The results obtained suggest that:

(1) Axotomy led to 16% neuronal loss in L4 and L5 DRGs 4 weeks after injury; administration of NT-3 systemically for 4 weeks prevented neuronal loss, but did not reduce neuronal apoptosis. The appearance of nestin- and β-III tubulin-immunoreactive cells in these DRGs suggests an axotomy-initiated replacement mechanism, which was enhanced by systemic NT-3 treatment.

(2) The changes of neurotrophin and neurotrophin receptor mRNA suggest a higher overall responsiveness of DRG neurons to neurotrophins after axotomy and NT-3 treament.

(3) Microarray data showed the up-regulation of a few genes relevant to neuronal lineage commitment of progenitor cells following axotomy and that some signalling pathways were activated after axotomy and NT-3 administration. NT-3 may stimulate axonal regeneration after axotomy.

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ABBREVIATION

BBB: blood-brain barrier

BDNF: brain-derived neurotrophic factor

BMP: bone morphogenetic protein

BMSC: bone marrow stromal cell

BrdU: bromodeoxyuridine

CaM: calmodulin

CaMK2: calmodulin-dependent protein kinase II

CCK: cholecystokinin

CGRP: calcitonin gene-related peptide

CNS: central nervous system

CNTF: ciliary neurotrophic factor

CRBP: cytosolic retinol-binding protein

CRE: cAMP response element

CREB: cAMP response element binding protein

CRLR: calcitonin receptor-like receptor

CV: conduction velocity

DREZ: dorsal root entry zone

DRG: dorsal root ganglia

EGF: epidermal growth factor

ELISA: enzyme-linked immunosorbent assay

EPSP: excitatory postsynaptic potential

Erk: extracellular signal-regulated protein kinase

bFGF: basic fibroblast growth factor

GABA: gamma-aminobutyric acid

GAP-43: growth-associated protein 43

GDNF: glial cell line-derived neurotrophic factor

GFAP: glial fibrillary acidic protein

GFP: green fluorescent protein

HSP27: heat shock protein 27

IB-4: Bandeiraea simplicifolia isolectin

IGF: insulin-like growth factor

IGFBP: insulin-like growth factor binding protein

IGIF: interferon-gamma inducing factor

IL-18: interleukin-18

ISEL: *in situ* end-labelling

JAK: *Janus protein tyrosine kinases*

JNK: jun-N terminal kinase

LIF: leukemia inhibitory factor

Mad: *Mothers against decapentapleic* (dpp)

MAP: mitogen-activated protein

MAP-2: microtubule-associated protein 2

MCP-1: monocyte chemoattractant protein-1

mRNA: messenger RNA

MW: molecular weight

NCSC: neural crest stem cells

Neu-N: neural-specific nuclear antigen

NFκB: nuclear factor-kappa B

NF: neurofilament

NGF: nerve growth factor

NPY: neuropeptide Y

NSC: neural stem cell

NT-3: neurotrophin-3

NT-4/5: neurotrophin-4/5

PACAP: pituitary adenylate cyclase-activating polypeptide

PDGF: platelet-derived growth factor

PI-3: phosphatidylinositol 3'-kinase

PLCγ: phospholipase C-γ

PNS: peripheral nervous system

PPT: preprotachykinin

RA: retinoic acid

RETL2: RET ligand 2

Rsk: ribosomal S6 kinase

SCG: superior cervical ganglia

SNAP: synaptosomal associated protein

SST: somatostatin

SSTR4: somatostatin receptor 4

SVZ: subventricular zone

SynGAP: Ras GTPase-activating protein

TGF: transforming growth factor

TNF: tumour necrosis factor

Trk: tropomyosin-related kinases

TUNEL: terminal deoxyribonucleotidyl transferase (TdT) uptake nick-end labelling

VIP: vasoactive intestinal peptide

WD: Wallerian degeneration

CHAPTER 1: REVIEW OF THE LITERATURE

1.1 Normal appearance and structure

1.1.1 Classes of sensory neuron in dorsal root ganglia

When dissected L4 and L5 dorsal root ganglia (DRGs) of rats appear as yellow fusiform swellings of the dorsal roots, measuring 2-3 mm in length. They are mainly composed of sensory neuron perikarya, each of them surrounded by satellite cells; in addition, they contain axons, myelinating and non-myelinating Schwann cells, fibroblasts, few mast cells, scanty extracellular matrix and blood vessels (Friede et al., 1967; Pannese et al., 1995; Lieberman, 1976). The capillaries penetrating the ganglia have fenestrated endothelia and come from the vascular plexus within its capsule (Jacobs, 1976). Due to the lack of known neuron-to-neuron connections and the apparent simplicity of the tissue, many studies have used DRG to investigate the effects of sensory nerve injury and they have also been my choice to study the injury-regulated genes against the background of constitutive gene expression.

The DRG neurons can be divided into subpopulations by biochemical, molecular or physiological phenotypes each of them thought to transmit different modalities of sensory information (Lawson, 1992). Commitment to different neuronal lineages occurs during neural crest cell migration (Rifkin et al., 2000); however the molecular signals that are responsible for this are starting to be understood. In this review, I will concentrate on the functional and biochemical aspects of adult lumbar DRG neurons as they have been extensively studied and are particularly relevant for my study, which deals with the interaction between DRG cells and neurotrophins.

Using histological DNA/RNA stains, such as cresyl fast violet and toluidine blue, to show Nissl (rough endoplasmic reticulum) substance clearly, DRG neurons were originally divided into large light cells (L neurons, 10-60 μm in diameter) with myelinated axons, which make up around 20-30% of the DRG neurons and the small dark cells (SD neurons, 10-25 μm in diameter) with unmyelinated axons (see Lieberman, 1976; Lawson, 1992; Tandrup, 1993, 1995). L neurons contain clumped Nissl substance, which leaves lightly staining spaces of cytoplasm filled with neurofilaments and microtubules. The darker staining of the SD neurons is due to the higher density of ribosomes in their cytoplasm with a more homogeneous distribution. Despite the labelling "Large" and "Small" there is a considerable overlap between these two groups (Lawson et al., 1984; Tandrup, 1993). L neurons express high levels of neurofilament protein, including the 200kD subunit NF-H, the 150-kD subunit NF-M and the 68-kD subunit NF-L. In both myelinated and unmyelinated axons, the axon calibre correlated well with the amount of neurofilaments (Friede and Samorajski, 1970). In addition, SD neurons contain peripherin, a type III intermediate filament protein. There is coexistence of peripherin and neurofilament-L in around 5.6% DRG neurons (Portier et al., 1983; Lawson et al., 1984; Goldstein et al., 1991). DRG cells that synthesise large amounts of neurofilament proteins have more neurofilaments in their axons as well as in their cell bodies, and have larger diameters of cell bodies and axons (Portier et al., 1983; Hoffman et al., 1987). In L5 DRG, around 70% of DRG neurons are SD neurons and approximately 28% are L neurons with 2% being unclassifiable (Tandrup, 1993). L4 DRG is probably similar, although there is no information about the distribution of these two types of neurons within other individual ganglia.

Anatomical and electrophysiological evidence for the correlation of SD neurons with unmyelinated axons and L neurons with myelinated axons in the periphery has been described (Yoshida and Matsuda, 1979; Sugiura *et al.*, 1988); however,thinly myelinated Aδ fibres may show features of either group, such as RT97 immunoreactivity (Lawson and Waddell, 1991). The majority of the small sensory neurons with unmyelinated axons are generally nociceptive (heat, cold, inflammation etc.); small, thinly myelinated axons are generally mechanosensory (light touch, vibration, high threshold mechanoreceptors). Those with myelinated axons are larger neurons; they are involved in heterogeneous functions such as proprioception, mechanoception and touch sensation (Lawson and Waddell, 1991, 1993). Peripherally, proprioceptive afferents terminate on muscle spindles (Ia afferents) and Golgi tendon receptors (Ib afferents) in the muscle and provide information about muscle length and tension to the CNS. Centrally, both Ia and Ib afferents send projections to the spinal cord and synapse with interneurons; some axons ascend in the spinal cord and finally synapse at the medulla. In adult rats, the expression of tropomyosin-related kinase (trk) family receptors in DRG neurons correlates well with functions of sensory neurons (McMahon et al., 1994). Most of the DRG proprioceptive neurons express trkC and only around 20% of them express trkA (McMahon et al., 1994).

1.1.2 Contribution of L4 and L5 DRGs to the sciatic nerve

The adult rat sciatic nerve at mid-thigh level, where we induced permanent damage by transection in this study, is composed of about 23,700-27,000 axons (Jenq and Coggeshall, 1985; Schmalbruch, 1986), including 19,000 sensory axons originating from around 10,500-11,000 ± 2,000 L4 and L5 DRG neurons, motor axons from 2,000 motor

neurons in spinal cord segments L3-L6 (Schmalbruch, 1986; Swett et al., 1986) and around 6,200 sympathetic axons (Schmalbruch, 1986). The total number of L4 and L5 DRG neurons in an adult rat is around 27,000 (Schmalbruch, 1987b; Groves et al., 1999; Groves et al., 2003), which is much more than the number of sensory neurons whose axons extend through the mid-thigh level; therefore the axons of remaining neurons may exit before they enter the sciatic nerve and go to areas such as the viscera, the pia (Risling et al., 1994), the muscles and the skin above the cut. It is calculated that mid-thigh sciatic nerve transection axotomises around 50-70% of L5 DRG neurons (Devor et al., 1985; Himes and Tessler, 1989). An increasing number of postnatal DRG neurons has been reported in 3 studies (Devor et al., 1985; Popken and Farel, 1997; Farel, 2002); therefore the ratio of right: left was used in my study to avoid the uncertainty of comparing the absolute number of neurons at different ages. However, the relative individual contribution of L4 and L5 DRG neurons to the sciatic nerve is unclear.

Using WGA-HRP/HRP labelling method, Swett et al. (1991) reported that 98.4 % of sciatic sensory axons are contributed to by L4 and L5 DRGs in the rat whereas L3 and L6 DRGs contribute 0.3 and 1.2% respectively. Therefore, L4 and L5 DRG neuron counts are pooled together in this study instead of looking at individual ganglion neuron numbers.

1.1.3 Interaction with other components in DRG

In DRG, sensory neurons are surrounded by a perineuronal microenvironment (Pannese et al., 1994), which mainly consists of satellite cells, Schwann cells and the extracellular matrix. The satellite and Schwann cells are known to provide trophic support to sensory neurons with which they are associated (Heumann et al., 1987), induce neuronal

maturation *in vitro* (Mudge, 1984) and affect the axon diameter (de Waegh et al., 1992). In turn, DRG neurons are also able to influence these supporting neuroglial cells, and regulate their proliferation, differentiation and maturation (Wood and Bunge, 1975; Jessen et al., 1987).

1.2 Characteristics of neurotrophins and their receptors

In mice and rats, four members of neurotrophin family have been identified: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5) (Levi-Montalcini and Cohen, 1960; Barde et al., 1982; Hohn et al., 1990; Berkemeier et al., 1991). Neurotrophin genes are translated into pro-proteins that are a little longer than 200 amino acids, which are then cleaved by proteases, such as plasmin and selective matrix metalloproteinases, and released as a mature C-terminal biologically active peptide. These form neurotrophin dimers by non-covalent bonds, slightly shorter than 120 amino acids long in mammals; approximately 50% of the amino acids are conserved in all neurotrophins. These factors act largely through trk family receptors, each of them expressed in a subpopulation of DRG neurons, with NGF interacting with its preferred receptor trkA, BDNF and NT-4 with trkB, and NT-3 with trkC. NT-3 also binds to trkA and trkB, although with significantly lower affinity, and NT-3 has been shown to initiate trkA signalling and cellular responses, and is required to maintain trkB-expressing neurons during neurogenesis in development (Clary and Reichardt, 1994; Davies et al., 1995; Farinas et al., 1998). Truncated isoforms of trkB and trkC have been characterised which lack most of the sequence for the cytoplasmic region, including the

protein tyrosine kinase domain, of the full-length isoforms (Klein et al., 1990a; Middlemas et al., 1991; Tsoulfas et al., 1993; Valenzuela et al., 1993).

The low affinity neurotrophin receptor $p75^{NTR}$ can also interact with all of these neurotrophins and regulate intracellular signalling pathways. The two classes of neurotrophin receptors, high- and low-affinity, are physico-chemically distinguished by their dissociation constants of $10^{-11}/10^{-10}$ MM and 10^{-9} M, respectively (Sutter et al., 1979). ProNGF also binds to $p75^{NTR}$ and trkA with higher and lower affinity respectively than that exhibited by mature NGF, and can induce apoptosis (Lee et al., 2001), but whether other pro-neurotrophins can activate $p75^{NTR}$ and trigger apoptosis is not well known.

These neurotrophins are secreted by target organs, internalised by interacting with trk receptors, and retrogradely transported to the cell soma, in which activating intracellular signalling pathways, such as the ras/raf/mitogen-activated protein (MAP) kinase pathways, the phosphatidylinositol 3'(PI-3)-kinase/Akt pathways and the phospholipase C-γ (PLCγ) pathway, regulate adult sensory neurons (Vetter et al., 1991; Obermeier et al., 1993; Baxter et al., 1995; Goldberg and Barres, 2000). NGF, BDNF and NT-3 mRNAs are also expressed in uninjured DRG neurons (Zhou et al., 1999b), and are essential for the survival and phenotypic differentiation of sensory neurons during development.

Most of the early studies on neurotrophins in development were performed in chicken and mouse. During embryonic development in these animals, neural crest cells migrate, proliferate, aggregate and coalesce lateral to the neural tube to form the nascent DRG. Neurons are then derived from neuronal crest stem cells in the DRG (Weston, 1963; Sharma et al., 1995), with larger diameter neurons, including proprioceptive neurons, born and differentiated prior to the smaller neurons (Lawson et al., 1974; Carr and Simpson,

1978); a majority undergo programmed cell death after the postmitotic stage, when differentiating neurons project axons toward their cutaneous, muscle and visceral targets and both birth and death of neurons happens simultaneously in nascent DRG (Farinas et al., 2002). In mice, null mutants of the genes encoding all neurotrophins and their receptors have been generated to analyse their effects on neuronal development (Snider and Silos-Santiago, 1996).

1.2.1 Early sensory development and neurotrophic hypothesis

The mouse DRG neurons derive from the neural crest cells that migrate to a dorsolateral position along the neural tube at E9 and coalesce into somite-related clusters to form the discernible ganglia at E10 (Farinas et al., 2002). Subsequent cell proliferation, studied by BrdU incorporation, and addition of new migrating neural crest cells result in the rapid enlargement of ganglia. Nearly all the DRG neurons are generated between E10 and E13 with the peak rate of neurogenesis between E12-E13 (Lawson and Biscoe, 1979). In the developing peripheral nervous system of mice, DRG neurons initiate axon elongation immediately after they are generated at as early as E10. Many neurons die shortly after their axons have reached their target fields, possibly because the limiting amounts of neurotrophic factors in target organs which new neurons innervate select only the specific neuronal subpopulations which obtain enough substance and eliminate the remaining through programmed cell death (Barde et al., 1989; Barde, 1994; Purves et al., 1996). This is known as the neurotrophic hypothesis and was formulated to account for the behaviour of sensory and motor neurones during the latter stages of development during which the total population was reduced by approximately 50%. However, the epidermis in trunk and

proximal limbs are innervated at around E13 and distal limbs are innervated at as late as E18; furthermore, trkC knockout mutants have elevated apoptosis in lumbar DRGs at E11 before their axons approach their target organs support (Coggeshall et al., 1994; Farinas et al., 1996). Therefore, the neurotrophic hypothesis can only describe a small part of neurotrophin action on sensory neurons; even during development neurotrophins have function that exceeds their role in assuring neuronal survival, and they may act on the differentiation of precursor cells and continue to act on sensory function in the adult (Farinas et al., 1996). The neurotrophic hypothesis implies that neurotrophins are derived from the target organ, but it has been shown that a broader distribution of these neurotrophins exists also in sensory nervous system (Shen et al., 1999a; Zhou et al., 1999b).

1.2.2 Nerve growth factor (NGF)

Nerve growth factor (NGF), the best characterised member of the neurotrophin family, was first identified by Levi-Montalcini (1960) and plays an important role in the development and survival of sympathetic and a subpopulation of sensory neurons in DRG (Levi-Montalcini and Cohen, 1960; Levi-Montalcini and Angeletti, 1963). It may function as a mediator of some persistent pain and controls the "activity" of trkA-expressing nociceptive neuron; trkA-IgG administration has been shown to produce a sustained thermal and chemical hypoalgesia (McMahon et al., 1995; Malcangio et al., 2000). Some of its actions are mediated by its high affinity receptor trkA, and it plays a role in the maintenance and regeneration of mature peripheral neurons (Kaplan et al., 1991). NGF also mediates its effects via the low affinity neurotrophin receptor $p75^{NTR}$ (Johnson et al., 1986). NGF is mainly synthesised in skin, a major target of nociceptive sensory neurons, and also

in a subpopulation of DRG neurons (Zhou et al., 1999b). At E11.5 in the mouse, NGF mRNA starts to be detectable in the surface ectoderm of the developing hindlimb (Schecterson and Bothwell, 1992; White et al., 1996). The expression of NGF transcripts increases from E13.5 to E15.5, when NGF is strongly expressed in the epithelium of the hindlimb epidermis and subjacent mesenchyme (White et al., 1996). Increased cell death from E11 to E13 in both NGF and trkA null mutant mice shows the synchronous onset of NGF and trkA survival dependence in the developing DRGs (Farinas et al., 2002).

In the adult, NGF mRNA expression can be detected in DRG (Shelton and Reichardt, 1986; Zhou et al., 1999b). The fact that antiserum to NGF does not result in further decrease of cell number in DRGs after postnatal day 2 shows that NGF is required for survival of lumbar DRG neurons mostly during development, but not in the adult (Tonra and Mendell, 1998). However, 5-week NGF antiserum administration starting from postnatal days 4 to 11 switches many Aδ- high nociceptive afferents to be high-threshold D-hair afferents, thus revealing the functional importance of NGF in neonatal rats (Lewin et al., 1992).

1.2.3 Brain-derived growth factor (BDNF) and NT-4/5

BDNF was first purified from mammalian brain (Barde et al., 1982) and its amino acid sequence shows about 40% homology with that of NGF. BDNF, which acts through the trkB and $p75^{NTR}$ receptors, is synthesised in a small subpopulation of sensory neurons, predominantly NGF-responsive, trkA-expressing neurons, both during development and adulthood. (Ernfors et al., 1990; Schecterson and Bothwell, 1992; Apfel et al., 1996; Michael et al., 1999); this neurotrophin may maintain the survival of a subpopulation of

sensory neurons through an autocrine or paracrine loop; in addition it is anterogradely transported, probably to provide trophic support for peripheral sensory organs and postsynaptic neurons within the spinal cord (Acheson et al., 1995; Zhou and Rush, 1996; Michael et al., 1999). At E11 in murine embryos, expression of BDNF mRNA can be detected in dermal mesenchyme, which is a target of sensory neurons, but not within the mesenchyme immediately adjacent to DRG cells (Schecterson and Bothwell, 1992). BDNF promotes the survival of a subpopulation of neurons at E11, but exerts no effects at E12-E18 *in vitro*; it appears to have survival promoting property at postnatal day 7 (P7), when 35% of DRG neurons in culture are rescued by BDNF (Lindsay et al., 1985; Acheson et al., 1995). NT-4/5, also acting through trkB, is a survival factor for a specialised type of mechanoreceptive neuron, called a D-hair receptor, in the adult DRG (Stucky et al., 2002).

1.2.4 Neurotrophin-3 (NT-3)

In mice, NT-3 mRNA is expressed in mesenchymal cells in the dermamyotome immediately adjacent to the DRG and in muscle precursors in the proximal limb bud between E10 and E12 before their axons have reached their peripheral targets (Farinas et al., 1996; White et al., 1996); later in development at E13.5, it appears in all developing paraxial and hindlimb muscle precursors along the growing nerves, and it is expressed in the intrafusal muscle fibres of the muscle spindles, Golgi tendon organs, Merkel cells and a subpopulation of DRG neurons after muscle spindle innervation (ElShamy and Ernfors, 1996; Farinas et al., 1996; White et al., 1996; Copray and Brouwer, 1997); knockout of NT-3 gene leads to the excessive death of proliferating sensory precursor cells as early as E11 (ElShamy and Ernfors, 1996; White et al., 1996), and prevents sensory precursor cells

from differentiating into NT-3 dependent neurons, instead overriding the G1 phase restriction point and dying by apoptosis in S phase (ElShamy et al., 1998). *In vitro*, E11 DRG neurons can survive without exogenous NT-3 for at least 1 day, but anti-NT-3 antiserum or NT-3 null mutation can lead to their death (Vogel and Davies, 1991; ElShamy and Ernfors, 1996); this shows that in early development DRG neurons may synthesise NT-3 to support their own survival. Not only affecting postmitotic neurons, NT-3 knock-out mice show premature differentiation of precursor cells between E11 and E12 without changes in rates of their proliferation or apoptosis, resulting in early depletion of the pool of precursor cells (Farinas et al., 1996). In migrating murine neural crest cells, trkC mRNA can be detected by radioactive *in situ* analysis (Tessarollo et al., 1993); however, these cells do not express detectable levels of any trk receptor protein due to proposed translational repression (Farinas et al., 1998). Therefore, actions of any neurotrophin on precursor cells are suggested to be indirect.

In NT-3 homozygous knockout mutant mice, around 60-70 % of DRG neurons are lost between E10.5 and E13, involving all subpopulations of neurons (Ernfors et al., 1994; Farinas et al., 1996), but trkC knockout mutant mice only results in 17-30% loss (Klein et al., 1994; Minichiello et al., 1995). These discrepancies suggest that NT-3 can support survival of embryonic sensory neurons via trkA or trkB signalling rather than through its preferred receptor trkC (Davies et al., 1995). However, when trkA and trkB are expressed in P12 cells, they can only be activated by NT-3 at concentrations 100-fold higher than their preferred ligands, NGF and BDNF (Ip et al., 1993; Farinas et al., 1998).

Application of exogenous NT-3 during the formation of sensory ganglia in chick embryos results in a marked decrease in neuronal numbers, possibly due to the premature

cessation of sensory precursor cell proliferation and initiation of differentiation (Ockel et al., 1996b; ElShamy et al., 1998). However, administration of NT-3 at later developmental stages increases neuronal number, presumably by preventing programmed cell death in DRG (Ockel et al., 1996a). In rats, administration of antiserum against NT-3 for 2 weeks from postnatal day 1 results in a 20% reduction of the number of L5 DRG neurons, a 25% decrease of the DRG weight, a 19% reduction in the number of myelinated axons in the saphenous nerve, and a significant shift toward the left in the size distribution histogram of neurons. However number or appearances of muscle spindles is not affected; it is not clear which subpopulation of sensory neurons is lost (Zhou et al., 1998).

In adult animals, NT-3 is synthesised both in muscle and skin (Copray and Brouwer, 1994; Kennedy et al., 1998; Botchkarev et al., 2000). In addition, NT-3 protein and mRNA is expressed in a subpopulation of large neurons, as well as in some small sensory neurons, without any detectable signal in non-neuronal cells in uninjured DRGs (Zhou and Rush, 1995; Zhou et al., 1999b).

1.2.5 Tyrosine kinase receptors: trkA, trkB and trkC

TrkA was originally identified as a proto-oncogene isolated from colon carcinoma cells, and was then found to mediate the biological effects of NGF (Martin-Zanca et al., 1986; Klein et al., 1991). Activation of high-affinity trk receptors ($K_D \sim 10^{-11}/10^{-10}$ M) by neurotrophins was later found to lead to the initiation of signal transduction cascades involved with proliferation, survival and differentiation of PNS and CNS neurons (Segal and Greenberg, 1996).

During development, neurogenesis in nascent DRGs is initiated shortly before E10 and shows a burst of neuron formation between E12 and E13 in mice (Farinas et al., 1996). Although mRNAs for trkB and trkC were observed in migrating neural crest cells in the mouse, their proteins seem to be synthesised concomitantly with neuronal differentiation (Klein et al., 1990b; Tessarollo et al., 1994). At E10.5-E11 in mice, when more than 60% of DRG cells are precursors, trkC protein is expressed by most of the DRG neurons, whereas trkB is present in a modest subpopulation of cells and only low levels of trkA are present (Farinas et al., 1998). Lack of neuronal precursors or neuronal loss at E10 in neurotrophin knockout mutant mice is consistent with the absence of expression of detectable trk proteins by migrating neural crest cells, which are therefore not affected by the lack of neurotrophins at that time point (Farinas et al., 1996; White et al., 1996; Liebl et al., 1997). In NT-3 null mutant mice at E11, a reduction of 35% and 68% of trkB and trkC - expressing neurons, respectively, was observed without any loss of trkA-expressing neurons (Farinas et al., 1998). Therefore, the direct activation of trkB by NT-3 *in vivo* has been suggested to explain their finding. From E11-13.5, trkA expression increases steadily and appears in the majority of cells; however trkC decreases to be only expressed in a minority of DRG cells (**Figure 1.1**) (Farinas et al., 1998). At E 11.5 in mice, trkA-positive axons start penetrating into the central region of the proximal hindlimb, approaching cutaneous targets in proximal hindlimb at E13.5, when the central projections are still limited to the dorsal root entry zone (White et al., 1996). By E15.5, some of the trkA-expressing axons reach the most distal portion of the hindlimb and the central axons also reach grey matter in the spinal cord (White et al., 1996). The onset of trkA and NGF dependence by E13.5 is synchronous and of equal magnitude, suggesting that the same

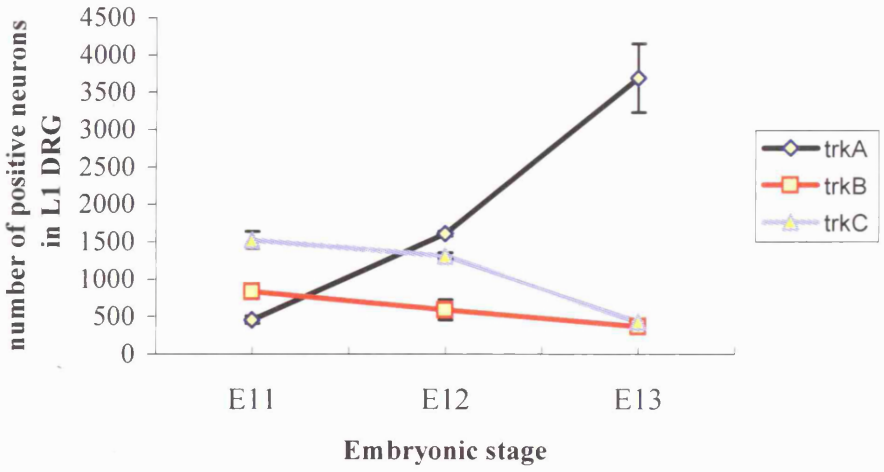
population of DRG cells requires both molecules (White et al., 1996). By E15, approximately 80% of DRG neurons express trkA mRNA and protein (White et al., 1996). After birth, trkC is mainly expressed in large DRG neuron. TrkB expression undergoes no obvious changes during this period of development, being expressed only by very few (6%) DRG neurons at E18 (Mu et al., 1993). The factors regulating the shift of trk expression during development are not clear. Using BDNF null mutant mice, it has been shown that endogenous BDNF is not required for the developmental induction of trkB expression and the onset of BDNF dependence in sensory neurons (Huber et al., 2000).

In adult, trkA is expressed in 35-45% of neurons, trkB in 20-33%, and trkC in 20-43% lumbar DRG neurons (Verge et al., 1992; McMahon et al., 1994), these receptors are also expressed on afferent fibres. Some of the trkC-expressing neurons are small cells, but primarily are medium to large sized cells (McMahon et al., 1994; Wetmore and Olson, 1995; Karchewski et al., 1999). Around 45% of the lumbar DRG neurons, mostly small neurons with unmyelinated axons that express calcitonin gene-related peptide (CGRP) and substance P, express trkA (McMahon et al., 1994; Averill et al., 1995). Chemical, thermal, and mechanical stimuli may all evoke pain when applied to the skin. Noxious stimuli are translated into electrical activity at the sensory endings of nerves and then the impulses are propagated throughout the sensory nervous system. CGRP and substance P, two important neuropeptides in the transduction of pain in the spinal cord, are synthesised in a subset of DRG neurons and released from the peripheral terminals of sensory nerves to induce inflammation by stimulating vasodilatation, inducing plasma extravasation and stimulating mast cell degranulation to release inflammatory mediators such as histamine and serotonin (Maggi et al., 1993).

Analysis of neuronal profiles shows that trkA, trkB and trkC can be expressed in neurons of all size categories. Co-localisation studies show that approximately 10% of DRG neurons co-express trkA and trkB mRNA; 18% coexpress trkB and trkC mRNA, and 19% coexpress trkA and trkC mRNA (McMahon et al., 1994; Wright and Snider, 1995). Localisation of all three trk mRNAs is seen only in 3-4% of neurons (Karchewski et al., 1999). Only 16%, 9% and 10% of the DRG population express one trk receptor (trkA, trkB and trkC respectively) to the exclusion of the others (Karchewski et al., 1999). The trkA-expressing neurons have a mean cross-section area 5-10% smaller than the mean value of the total population in lumbar DRGs; however, trkB and trkC-expressing cell profiles have a 5% and 25-50% larger than average area, respectively (Bergman et al., 1999).

In the DRGs of NGF or trkA knockout mutant mice, approximately 70-82 % of the DRG neurons are missing, including those which express trkA postnatally (Crowley et al., 1994; Smeyne et al., 1994; Silos-Santiago et al., 1995) and the subpopulation of non-peptidergic small-diameter neurons, which bind the Bandeiraea simplicifolia isolectin (IB4), with unmyelinated axons which may mediate non-nociceptive thermal and low threshold mechano-receptive stimuli (Silos-Santiago et al., 1995), but it is clear that many of them express TRP V1 and are therefore likely to be nociceptors. The latter neurons, accounting for a third of adult DRG neurons, depend on NGF transiently during embryonic development, and then switch their dependence from NGF to glial cell line-derived neurotrophic factor (GDNF) postnatally (Bennett et al., 1996b; Molliver et al., 1997). These cells start to express Ret during perinatal development (Molliver et al., 1997), which is part of a common receptor complex for GDNF and neurturin (Bennett et al., 1998).

Physiologically, conduction velocity (CV) of sensory axons significantly slowed by 1 week after peripheral axotomy, affecting all fibre sizes, especially those of larger diameter (Munson et al., 1997). Treatment with trkB-IgG or trkC-IgG fusion proteins at the dose of 12 μg/d into the region of the gastrocnemius muscles for 2 weeks in normal rats to sequester BDNF and NT-3 does not reduce CV of gastrocnemius sensory neurons, but a higher dose of trkC-IgG at 60 μg/d results in a significant reduction in CV (Munson et al., 1997). NT-3 administration to the proximal stump of tibial nerve (60 μg/d) for two weeks after 3 week axotomy partly rescues the decrease of CV. These results imply that NT-3 continues to play a role in physiological functions of a subpopulation of DRG neurons in adult rat (Munson et al., 1997).



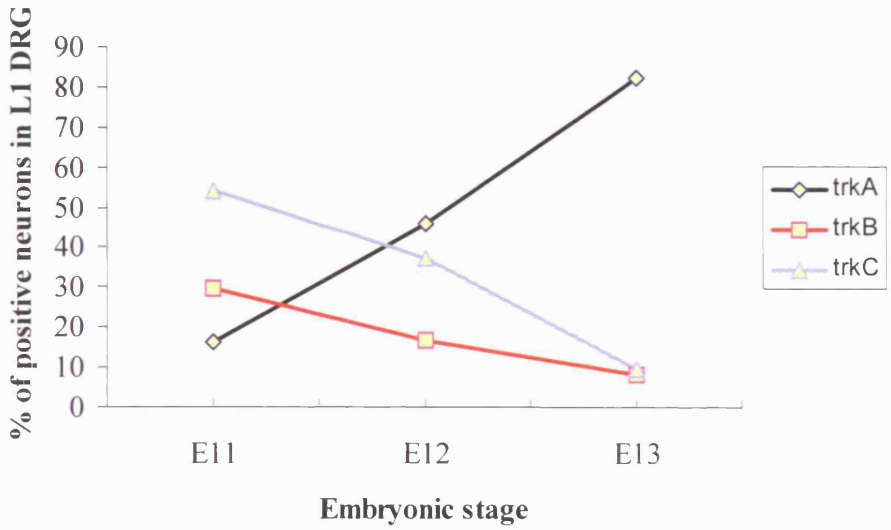


Figure 1.1: Numbers and percentages of trkA, trkB, trkC protein-expressing neurons in early L1 DRG in mice (Farinas et al., 1998). Virtually all cells expressing any trk receptor were also labelled by antibodies to the 150kDa neurofilament, and no proliferating precursors, labelled by bromodeoxyuridine (BrdU), coexpressed a trk receptor.

1.2.6 Intracellularly-spliced trk receptor isoforms

By alternative splicing, the trkB and trkC loci additionally encode a few isoforms which lack the intracellular catalytic tyrosine kinase domain, but either include instead alternative cytoplasmic domains, or have insertions in the tyrosine kinase domains (Klein et al., 1990a; Tsoulfas et al., 1993; Valenzuela et al., 1993). These, named "non-catalytic" or "truncated", domains are highly conserved across species; the intracellular truncated domains in trkB isoforms are different from those in trkC isoforms (Garner and Large, 1994; Armanini et al., 1995). 8 isoforms of trkC have been identified in the chick and mammal (Tsoulfas et al., 1993; Valenzuela et al., 1993; Garner and Large, 1994), and

different isoforms may mediate distinct activities in immature and mature DRGs. Although no signalling molecules downstream have been identified, the importance of these truncated isoforms in signalling pathways is suggested due to the highly conserved intracellular domains in human, mouse, rat and chicken (Garner and Large, 1994; Baxter et al., 1997). The truncated trkC isoforms are widely expressed in normal mouse development, throughout the CNS and PNS, including the DRGs. Overexpression of truncated trkC transgene in the mouse results in DRG neuronal loss between E11.5-E13.5 as cells are prematurely driven out of the cell cycle with differentiation of neural progenitor cells; this effect resembles that of early depletion of precursor cells in the NT-3 null mutants. The overexpressed truncated trkC may promote neural differentiation, with the collaboration of $p75^{NTR}$, in some cells (Hapner et al., 1998; Palko et al., 1999). Other isoforms with peptide insertions in the tyrosine kinase domain are still phosphorylated by their ligands, but are limited in their signalling potential (Garner and Large, 1994). Targeted deletion of the entire gene for trkC in mice leads to a more severe defect of the nervous system than in mice in which only the catalytic isoform has been deleted (Liebl et al., 1997; Tessarollo et al., 1997). The exact functions of these trk isoforms during neural development and in the adult are not completely known.

Outside the nervous systems, the trk receptors, mostly truncated isoforms, are also expressed during development, in lymphocytes, spleen, tooth, salivary gland, lung, heart, thyroid gland, kidney, muscle, testis and hair follicle, suggesting that they may not be transducing neurotrophin signals but rather acting as scavenger or dominant negative receptors (Middlemas et al., 1991; Tessarollo et al., 1993; Eide et al., 1996; Menn et al., 1998). Truncated trkC isoform is important in the morphogenesis of the cardiac outflow

tract, and the differentiation, maintenance and function of monocytes, lymphocytes and mast cells (Burgi et al., 1996; Hiltunen et al., 1996; Levi-Montalcini et al., 1996); indeed NT-3 knockout mutant mice were found to have cardiac defects (Donovan et al., 1996; Srivastava and Olson, 1996).

1.2.7 Extracellularly-spliced trk receptor isoforms

Alternative splicing of an exon can occur in the extracellular domain of trkA and trkB, resulting in isoforms (Barker et al., 1993). These are only present in chick and man, not in rodents (Strohmaier et al., 1996). The trkB isoform, which can bind BDNF with similar affinity to its full-length receptor, has reduced affinities for NT-4/5 and NT-3; in contrast, the trkA isoform has a higher affinity for NT-3 (Clary and Reichardt, 1994).

1.2.8 Low-affinity neurotrophin receptor: $p75^{NTR}$

The low-affinity neurotrophin receptor $p75^{NTR}$, which is the prototypic member of the tumour necrosis factor (TNF) receptor family, is a transmembrane glycoprotein of 75k Da which exhibits lower affinity binding ($K_D=10^{-9}$ M) for all neurotrophins than their preferred trk receptors. Its expression is widespread in most dividing progenitor and post-mitotic neurons in developing DRG with a peak at E11.5-E12.5 in mice (Schecterson and Bothwell, 1992; Snider et al., 1992). It does not have a known intrinsic ligand-inducible enzymatic function, but it is able to induce signalling independently and modify the binding and signalling capabilities of trk receptors (see Roux and Barker, 2002). The coexpression of $p75^{NTR}$ exerts conformational changes in trkA and enhances the affinity of trkA-expressing neurons for NGF binding at low concentrations (Hempstead et al., 1991;

Hantzopoulos et al., 1994). $p75^{NTR}$ affects the retrograde axonal transport of neurotrophins and may be functionally important during normal development, when tissue levels of neurotrophins are a limiting factor (Bothwell, 1995; Curtis et al., 1995). NGF and BDNF binding is more affected by $p75^{NTR}$ expression than NT-3 binding. On the majority of migrating neural crest cells, $p75^{NTR}$, both in mRNA and protein, is broadly and strongly expressed (Rifkin et al., 2000).

In vitro studies show that $p75^{NTR}$ suppresses the ability of trkA to respond to NT-3 (Clary and Reichardt, 1994; Mischel et al., 2001); NT-3 only activates trkA signalling in PC12 rat pheochromocytoma cells, which extend neurites and differentiate into neuron-like cells upon exposure to NGF (Greene and Tischler, 1976), when $p75^{NTR}$ levels are reduced (Benedetti et al., 1993). In $p75^{NTR-/-}$ $NGF^{+/-}$ mutant mice, endogenous NT-3 can activate trkA receptors to compensate for reduced levels of NGF in sympathetic neurons and there is no reduction of neuron number in superior cervical ganglia (SCG) of these mice (Brennan et al., 1999). However, controversial findings from two groups leaves open the question of whether the coexpression of $p75^{NTR}$ and trkB decreases trkB phosphorylation in response to NT-3 or other neurotrophins (Bibel et al., 1999; Vesa et al., 2000).

In adult rodents, approximately 50% of trkC-expressing neurons express $p75^{NTR}$ (Wright and Snider, 1995), and 55-79% of neurons of all sizes express $p75^{NTR}$ (Zhou et al., 1996; Bergman et al., 1999; Karchewski et al., 1999). $p75^{NTR}$ is also present in some satellite cells (Zhou et al., 1996). Co-localisation of $p75^{NTR}$ with trks is higher during development, when both trkC and $p75^{NTR}$ receptors are more widespread and $p75^{NTR}$ expression reaches a peak at E11.5-E12.5 (Schecterson and Bothwell, 1992). $p75^{NTR}$ receptor is not essential for the development of proprioceptive neurons to fast, but not slow

(e.g. soleus muscle), hindlimb muscle in mice when NT-3 is expressed at normal levels. However, when NT-3 expression decreases, $p75^{NTR}$ may act in synergy with NT-3 to enhance the survival of proprioceptive neurons (Fan et al., 1999). Other potential roles for $p75^{NTR}$ include the modulation of trk signalling capabilities (Verdi and Anderson, 1994), inhibition or promotion of apoptosis (Rabizadeh et al., 1993), and activation of a signal transduction pathway by neurotrophins that involves the hydrolysis of sphingomyelin (Dobrowsky et al., 1995). This versatile receptor uses different ligands in distinct cell types, embryonic development, or following injury to mediate cell motility, proliferation, survival and death (Ibanez, 2002; Roux and Barker, 2002).

1.2.9 Spliced $p75^{NTR}$ isoform

Alternative splicing of exon III in the $p75^{NTR}$ gene, which encodes the extracellular cysteine-rich domains essential for the binding of neurotrophins, occurs in human, mouse, rat and chick (Dechant and Barde, 1997). This short isoform, called s-$p75^{NTR}$, shares identical transmembrane and cytoplasmic domains with the full-length $p75^{NTR}$ and may act as a modulator of the full-length receptor.

1.2.10 Molecular interactions between trk receptors and $p75^{NTR}$

Biochemical experiments show that neurotrophin receptors may form three kinds of complexes: homodimers of trk receptors, homomeric $p75^{NTR}$ complexes and heteromeric complexes containing both trk and $p75^{NTR}$ receptors. These complexes coexist in some neurons and transduce independent, synergistic or antagonistic signalling. *In vitro* studies indicate that $p75^{NTR}$ interacts differently with different types of trk receptors. Co-

expression with $p75^{NTR}$ in PC12 rat pheochromocytoma cells enhances NGF signalling via trkA by increasing trkA tyrosine autophosphorylation, and desensitises trkA to NT-3 (Benedetti et al., 1993; Verdi and Anderson, 1994). However, $p75^{NTR}$ has either a negative or positive effect on BDNF and NT-4/5 signalling via TrkB depending on the cellular system (Bibel et al., 1999; Vesa et al., 2000). Coexpression of $p75^{NTR}$ does not change the autophosphorylation of trkC by binding of NT-3 in an MG87 fibroblast cell line (Vesa et al., 2000). In addition to the interactions between trk receptors and $p75^{NTR}$ at the level of physical association of the proteins, interactions between $p75^{NTR}$ and trk signalling cascades are also indicated (Bilderback et al., 2001).

1.2.11 Interaction of neurotrophins with their receptors

After binding to trk receptors, neurotrophins and their phosphorylated receptors are taken up via clathrin-dependent mechanisms into intracellular small vesicles, forming complexes. Endocytosed complexes, which differ according to the cell-type and neurotrophin involved, are retrogradely transported to cell body and induce signal transduction (Ehlers et al., 1995; Bhattacharyya et al., 1997; Grimes et al., 1997). $p75^{NTR}$ may modulate trkA internalisation and change its signalling spectrum (Gargano et al., 1997). Other than being retrogradely transported, the receptors in complexes may reappear back to the membrane surface or be degraded. Therefore, the trk receptors that respond to neurotrophins can either be newly synthesised or recycled from the signal transducing vesicles (Eveleth and Bradshaw, 1988).

1.2.12 Signal transduction

The effects of neurotrophins binding to their receptors are believed to involve the activation of intracellular protein kinases. Multiple parallel signalling pathways have been found to be involved in neuronal survival. Particularly, three intracellular pathways seem to be important in mediating survival, differentiation and neurite growth of sensory neurons (Obermeier et al., 1993; Baxter et al., 1995; Goldberg and Barres, 2000):

1) The ras/raf/mitogen-activated protein (MAP) kinase pathway

2) The phosphatidylinositol 3'-kinase (PI-3 kinase)/Akt pathway

3) The phospholipase C-γ (PLCγ) pathway

MAP kinase signal transduction seems essential for NGF-stimulated outgrowth of adult DRG nociceptive neurons (Thomas et al., 1992; Jaiswal et al., 1994) and this effect is blocked by PD98059, which inhibits the MAP kinase activator MEK (Wiklund et al., 2002). In contrast, inhibition of MAP kinase enhances NT-3-stimulated outgrowth, which suggests differences in the trkA and trkC intracellular signalling pathways.

In the MAP kinase pathway, activated trk receptors on cell bodies induce GTP-loading and activation of Ras. Ras-GTP recruits a three-tiered enzyme cascade in which a Raf phosphorylates and activates Erk-1/2, which then translocates from the cytoplasm to the nucleus and activates ribosomal S6 kinase (Rsk). Activated Rsk then phosphorylates the transcription factor Ca2+/cAMP response element binding protein (CREB) at Ser 133 subsequently activating cAMP response element (CRE)-mediated gene expression, leading to alterations in gene expression (English et al., 1999; Finkbeiner, 2000; Chang and Karin, 2001). Rsk also phosphorylates Bad, which is a member of bcl-2 family that blocks cell survival by binding to another member of bcl-2 family, Bcl-$_X$L, in its unphosphorylated form, to inhibit its pro-apoptotic activity. In addition to Rsk, other neurotrophin-induced

CREB kinases have been identified, including MAPK-activated protein kinase-2, Ca^{2+}- and calmodulin-dependent kinase IV and protein kinase B (Finkbeiner, 2000). Ras activity is necessary for the survival of sensory neurons (Borasio et al., 1993), but the activities of its downstream effectors, Raf, Mek and Erk are not (Creedon et al., 1996). The Erk-5 pathway is also activated by neurotrophins and leads to CREB activation. These MAP kinases activate different groups of transcription factors; Erk-1/2 phosphorylates Elk-1 and c-Myc, whereas Elk-5 activates MEF2. However, using neuron culture in compartmented chambers with the cell body in one chamber and axon terminals in an adjacent chamber, neurotrophin stimulation of axon terminals resulted in localised activation of Erk1/2 and Erk5, but only activated Erk5 mediates retrograde signalling to the nucleus, which also leads to nuclear translocation of Erk5, phosphorylation of CREB, activation of MEF2 transcription factor and enhanced neuronal survival (Mao et al., 1999; Watson et al., 2001). In PC12 cells, the Ras cascade is necessary and sufficient for neurite outgrowth induced by NGF, but the cascade mediating differentiation of primary neurons from PC12 cells is still not clear (Creedon et al., 1996).

The PI-3 kinase/Akt intracellular pathways regulate NGF-stimulated neuronal survival (Yao and Cooper, 1995). PI-3 kinase is composed of a SH2-containing 85 kDa regulatory subunit and a 110kDa catalytic domain. Activated PI-3 kinase catalyses the formation of lipid second messengers, which activate the serine-threonine kinase Akt (Burgering and Coffer, 1995; Franke et al., 1997). Akt leads to cell survival via two possible pathways. One is through phosphorylation of Bad, which decreases its capability to bind to bcl-2 and bcl_xL and that promotes cell survival (Finkbeiner, 2000); another possible mechanism includes inactivation of caspase-9 (Yao and Cooper, 1995). PI-3

kinase activity is also required for neurotrophin-induced survival of sensory neurons (Crowder and Freeman, 1998), consequently it has been proposed that the PI-3 kinase cascade is downstream of Ras in primary sensory neurons (Rodriguez-Viciana et al., 1994). Both the MAP kinase and PI-3 kinase pathways converge on two proteins, Bad and CREB, to inhibit apoptosis and promote survival.

Activation of trks also triggers other signalling pathways such as the PLCγ pathway (Vetter et al., 1991). PLCγ binds to the phosphorylated trk receptors through src-homology domain 2 (SH2) and catalyses the breakdown of phosphoinositol 4,5-bisphosphate to diacylglycerol and inositol triphosphate, which then activate protein kinase C and also lead to an increase of intracellular calcium.

It is still an open question whether all of the neurotrophins signal through conserved intracellular cascades and if the cross talk between trk receptors and $p75^{NTR}$ determines the intracellular signalling mechanism. $p75^{NTR}$ has been shown to signal independently through activation of a sphingomyelin-based pathway to activate nuclear factor-kappa B (NFκB), jun-N terminal kinase (JNK), and ceramide to induce cell apoptosis (Carter et al., 1996; Casaccia-Bonnefil et al., 1996), although the signalling appears to be inhibited by trkA activation (Dobrowsky et al., 1995).

A limitation of the studies on signal transduction is that most of the biochemical studies of trk-related signalling pathways were performed in PC12 cells, instead of primary sensory neurons. PC12 cells are derived from a rat pheochromocytoma and are used as a model of a neuronal system because NGF induces differentiation of PC12 cells to resemble neurons, stimulates PC12 cells to stop division, extend neurites, express neuronal markers and become electrically excitable, and differentiated PC12 cells undergo apoptosis in

serum-free conditions without NGF (Tischler and Greene, 1975; Greene and Tischler, 1976; Greene, 1978). It is not clear how relevant PC12 studies are to events in true neurons. How neurotrophins exert their effects via their receptors in intact or injured DRG is not clear.

1.3 Morphological changes following axotomy

Axonal damage by crush, axotomy, ischemia, or inflammation leads to interruption of axonal integrity and initiates Wallerian degeneration in the distal stump (WD). This is characterised by axon degeneration, myelin sheath detachment and degradation, activation and proliferation of Schwann cells, to form bands of Bungner, and invading macrophages (Salzer et al., 1980). WD begins with degradation of axoplasm and axolemma induced by the activation of axonal proteases and calcium influx (Schlaepfer and Bunge, 1973; George et al., 1995). The proliferation of Schwann cells continues for approximately 2 weeks, eventually forming conduits that guide the regenerating axons to their peripheral targets and are a source of neurotrophic factors, such as NGF, BDNF and ciliary neurotrophic factor (CNTF) (Heumann et al., 1987; Acheson et al., 1991; Sendtner et al., 1992; Son and Thompson, 1995). In the proximal stump, the axons degenerate retrogradely to the first node of Ranvier and subsequently start to form regenerating neuronal sprouts within a few hours (Wong and Mattox, 1991).

1.3.1 Neuronal loss

In the peripheral nervous system, nerve transection (neurotmesis) and nerve crush are different models used to study the injury-induced degeneration and regeneration

responses. There are many reports of DRG neuron death after peripheral nerve lesion in adults, but the results are quite variable (Table 1.1). Nerve transection results in loss of continuity of all nerve elements; on the other hand, in crush injuries the integrity of the basal lamina of nerve fibres is maintained. It has been shown that crushing of the peripheral axons results in less severe neuronal loss in the affected ganglia than permanent transection because of highly effective regeneration due to continuity of basal laminae (Risling et al., 1983; Swett et al., 1995; Groves et al., 2003); crush-related functional deficit can completely recover in 2-3 months (Bridge et al., 1994). In addition to the type of nerve damage, the distance from the lesion to the DRG is also a factor in the rate and magnitude of neuronal death, which may be related to the loss of larger amount of axoplasm and the decreased trophic support from the proximal segment of injured nerve (Lieberman, 1974; Ygge, 1989; Shi et al., 2001).

Table 1.1: DRG neuron loss after nerve lesions in adult mammals

Study	Animal	Nerve damaged	Ganglia examined	Survival time	DRG cell loss
Risling et al., 1983	Cat	Sciatic	L7	35, 90 &190 days	30% at 30 days, 10% at 190 days
Ygge, 1989	Rat	Sciatic	L4/5	120 days	27% proximal lesion, 7% distal lesion
Swett et al., 1995	Rat	Sciatic, common peroneal or sural	Lumbar	15-187 days	Essentially none except 19% after peroneal crush
Vestergaard et al., 1997	Rat	L5	L5	4-45 days	35% by 45 days
Groves et al., 1997	Rat	Sciatic	L4/5	30-180 days	5.4% at 30 days, 17% at 90 days, 14% at 180 days
Tandrup et al., 2000	Rat	Sciatic	L5	14-224 days	6% at 14 days, 14% at 56 days, 37% at 224 days
Shi et al., 2001	Mouse	Sciatic	L5	7-28 days	24% at 7 days, 54% at 28 days

McKay Hart et al., 2002	Rat	Sciatic	L4/5	4-180 days	15% at 7 days, 35% at 60-180 days
Groves et al., 2003	Rat	Sciatic	L4/5	30-90 days	7.2% at 10 days, 2.5% at 90 days

Peripheral axotomy results in greater and more rapid neuronal loss in neonatal DRG than in the adult, possibly due to the higher dependence of neonatal neurons on target-derived trophic factors (Aldskogius and Risling, 1981; Risling et al., 1983; Bondok and Sansone, 1984; Schmalbruch, 1987a). In the axotomised adult DRG, different animal models, different injury types and locations and times and counting techniques accounted for the wide range of 7-50% of neuronal loss (Groves et al., 1997; Tandrup et al., 2000; McKay Hart et al., 2002). Transection with ligation of adult rat spinal nerve produced around 35% L5 DRG neuronal loss by 45 days (Vestergaard et al., 1997).

Whether there is a preferential type of primary afferent neurons lost after peripheral axotomy is still controversial. Some studies demonstrated a selective loss of small diameter neurons (Ranson, 1909; Cavanaugh, 1951; Rich et al., 1989; Vestergaard et al., 1997); however others found loss of larger neurons (Bondok and Sansone, 1984), or no selective effects (Ygge and Aldskogius, 1984; Arvidsson et al., 1986). Possible explanations include differences in the type and/or the lesion, age of the animals when the procedure was performed, the survival time before the neuronal number was counted and the method used to estimate sizes.

1.3.2 Stereological method for neuronal counting

Stereology was developed to avoid the biases inherent to serial reconstructions; in particular one stereological technique, the physical disector, was used in this study to estimate neuron number in each ganglion (Sterio, 1984; Gundersen et al., 1988; Williams

and Rakic, 1988). Stereological counting usually involves either the optical disector method, which counts particles in a three dimentional counting frame on thick tissue sections, or the physical disector method, which counts particles in one of two adjacent thin serial sections (3-4 μm in this study). Both make no assumptions about the size, shape or distribution of particles (such as neurons in this study) and avoid the bias derived from multiple or split nucleoli or nuclei. Compared to serial reconstruction technique, in which nuclei or nucleoli of neurons are counted in selected serial sections of ganglia and multiplied by the total number of serial sections with the application of a correction factor (Cavanaugh, 1951; Ygge et al., 1981; Himes and Tessler, 1989), the stereological method produces accurate reproducible estimations of neuron numbers in DRG (Coggeshall, 1992; Tandrup, 1993, 1995; Groves et al., 1997; Groves et al., 1999; Groves et al., 2003).

However, comparing absolute numbers of DRG neurons will require larger groups of animals due to high degree of variation between animals. Counting the neuron numbers in both L4 and L5 DRGs should also be used.

1.3.3 Neuronal apoptosis

The early perikaryal response to axotomy seen in nerve cells including DRG neurons is known as chromatolysis (Grafstein, 1983). This is characterised by the redistribution of Nissl substance from the central region of the perikaryon to the periphery, the displacement of nuclei to the periphery of the perikaryon, indentations of the nuclear membrane and alteration of neuronal shape. Chromatolysis is observed in affected neurons 24-48 hours after axonal damage, a time that depends on the distance of the injury from the DRG, and persists for 2-3 months after permanent axotomy (Pannese, 1963; Lieberman,

1974). After transient axotomy (axonotmesis) the chromatolytic appearance disappears as axons regenerate and contact their target.

Chromatolysis is not a process leading invariably to death of the cell. It is associated with increased RNA and protein synthesis and is primarily a regenerative response (Kreutzberg, 1996). Apoptosis, also known as programmed cell death, was first described by Kerr et al. in 1972 as a form of cell death followed by the disposal of unwanted cells during embryogenesis, metamorphosis, normal cell turnover as well as in some pathological situations (Steller, 1995; Nicholson, 1996). Unlike necrosis, apoptosis is an active process and is important in maintaining the integrity and homeostasis of multicellular organisms. During necrosis, synthetic functions suddenly stop and disruption of the plasma membrane leads to the influx of water and swelling of cells and organelles, rapidly followed by loss of the cell contents and cell disintegration (Majno and Joris, 1995). In contrast, apoptosis involves a process of chromatin condensation, internucleosomal DNA cleavage, blebbing of cell membrane, destruction of the nuclear membrane, condensation of cytoplasm, and finally the formation of membrane-bound apoptotic bodies (Majno and Joris, 1995; Hacker, 2000). Without any apparent breach of the cell membrane, the final products are finally phagocytosed without inducing inflammatory reaction or damaging adjacent tissues (Hacker, 2000). Apoptosis-inducing factors include damage to DNA by UV or γ-irradiation, oxidative damage, chemotherapeutic drugs, heat shock, withdrawal of growth factors and exposure to certain cytokines such as TNF-α and transforming growth factor (TGF)-β.

In the last few years, many of the molecules that participate in the execution of apoptosis have been identified. Various pro-apoptotic signals operate through two principal

pathways which use caspase-8 and caspase-9 to mediate the signalling pathways (see Vaughan et al., 2002). Caspase-8 is activated by stimuli through the cell membrane death receptor followed by recruitment of the Fas associated death domain (FADD), and caspase-9 is activated by the release of cytochrome c from mitochondria triggered by other stimuli, including radiation and cytotoxic drugs. Subsequently, the key regulator, p53, may activate caspase-3, -6, or -7, which are synthesised within cells as catalytically inactive pro-caspases and cleaved to active proteases in response to an apoptotic signal, and are activated by both apoptotic pathways (Nicholson and Thornberry, 1997; Slee et al., 2001). In particular, caspase-3 alone appears the last caspase to be activated for completing the final stage of apoptosis. Both immediate caspase-mediated destruction and components further downstream may cleave the proteins involved in maintenance of cellular function and structural proteins and lead to cell destruction (Nicholson and Thornberry, 1997). During apoptosis, a few proteins have been found to be able to modulate the processing of signals (see Vaughan et al., 2002); these include the Bcl-2 family which are involved in the suppression of cytochrome c release, FLICE-like inhibitory protein (FLIP)'s binding to FADD, and Inhibitors of Apoptosis Proteins (IAP's) suppressing caspase-9 and caspase-3 directly.

With regard to the correlation between apoptosis and neurotrophins, it is known that NT-3 and NGF knockout mutants show higher expression of activated caspase-3 and caspase-9 (Farinas et al., 2002), which are also activated in embryonic DRG during normal development, indicating the involvement of these effectors in neurotrophin deprivation-induced apoptosis (Yuan and Yankner, 2000). Caspase-3 inhibition rescues retinal ganglion cell apoptosis following axotomy and reduces ischaemia-induced hippocampal cell loss

(Chen et al., 1998; Kermer et al., 1998), but only slows the progress of neonatal motoneuron apoptosis following axotomy (Vanderluit et al., 2000). Immunostaining by antiserum against the active caspases in apoptotic pathway has been used to identify apoptotic cells in rats (Vanderluit et al., 2000).

Apoptotic neurons have been described in axotomised DRG (Ekstrom, 1995; Groves et al., 1997, 1999; Leclere et al., 1998; McKay Hart et al., 2002), and as early as 24 hours by McKay Hart et al. (2002), who used a staining technique named terminal deoxyribonucleotidyl transferase (TdT) uptake nick-end labelling (TUNEL) to label and visualise apoptotic cells (Gavrieli et al., 1992). In DRG, the peak was seen at 2-3 weeks and continued for at least 6 months after axotomy (Groves et al., 1997; McKay Hart et al., 2002). The TUNEL technique uses enzymatically mediated polymerisation of labelled nucleotides onto the free 3'-OH DNA ends of DNA strand breaks, which results from DNA digestion by calcium-activated endonuclease, followed by the detection of incorporated nucleotides by a secondary antibody; therefore DNA fragmentation happening in apoptosis can be detected by using TUNEL stains (Charriaut-Marlangue and Ben-Ari, 1995; Sanders and Wride, 1996; Yuan and Yankner, 2000). However, similar features have been shown to be non-specific for apoptosis as they can also be observed and stained in necrosis. *In situ* end-labelling technique (ISEL) is another technique used to detect single-stranded DNA existing in these two processes.

Although the methods introduced above are popular as tools to identify apoptotic cells, morphological criteria alone are well established and used to detect apoptosis in studies (Tong et al., 1996; Groves et al., 1997); only rarely is there ambiguity in identifying apoptotic DRG neurons in well-perfused tissue. It has been shown that only a

subpopulation of axotomised neurons dies after peripheral axotomy (Groves et al., 1997; Tatton et al., 1998; Tandrup et al., 2000; McKay Hart et al., 2002); however the factors which ultimately determine whether a specific subpopulation of neurons survives or dies after an injury are not understood.

In contrast to transection of peripheral axons, axotomy of dorsal roots, the centrally directed axons, of DRG neurons does not lead to an observable cell death in DRGs, and results in no chromalytic reaction in the affected sensory neurons (Lieberman, 1974; Titmus and Faber, 1990).

In my study, I proposed to use caspase-3 immunoreactivity and morphological criteria to characterise neuronal apoptosis. Activation of caspase 3 appears to be a key event in the execution of apoptosis in the central nervous system and has been observed in neurons undergoing apoptosis following CNS infarct (Charriaut-Marlangue 2004); activation of caspase 3 in other conditions has not been reported.

1.3.4 Ganglion volume and neuronal size profiles

Axotomy produces a reduced volume of the ipsilateral L4 and L5 DRGs when compared to the contralateral side (Rich et al., 1987). This decline in volume starts 1 week after axotomy and reaches a plateau by 2 months. The decrease in ganglion volume following peripheral axotomy reflects the neuronal loss and volume reduction of surviving neurons in injured ganglia. Previous studies have described the increase in rat L4 and L5 DRG volume by approximately 27% from birth to its maximum at 4 months of age or older (Bergman and Ulfhake, 1998; McKay Hart et al., 2002), and there is a general increase of neuron size when the neuronal profiles in postnatal day (P) 100 are compared to those in P1

rats (Farel, 2002). The degree of shrinkage of DRG neurons is to some degree related to the distance from the transection site to the DRG: Vestergaard et al. (1997) reported reduction of mean neuronal volume by 33% by 4 days after L5 spinal nerve transection, which persisted for at least 6 weeks, and both L and SD cells showed similar degree of shrinkage; Rich et al. (1987) reported a decrease of neuronal area by 19% at 3 weeks and 16% by 6 weeks after sciatic nerve transection at the level of the tendon of the obturator; Bergman et al. (1999) described a 10% decrease of cross-sectional cell profile area one week after mid-thigh sciatic transection. These authors used different methods to measure neuronal volume. In my study, I compared cross-sectional cell profile in the injured ganglia to that in the contralateral L4+L5 DRGs in each animal, minimising the factor of possible neuronal sized change during tissue processing.

1.3.5 Reactions of satellite cells

Satellite cells have apparently slightly flattened nuclei, flattened cell bodies and narrow, laminar processes, embracing DRG neurons (Bunge et al., 1967). The satellite cells make their appearance in the DRG after neuroblasts (Pannese, 1974), and one of their supposed roles is to regulate ion concentration in the microenvironment of neurons (Pannese, 1981). Recently, Pannese et al. (2002) reported immunoreactivity for trkA and $p75^{NTR}$ in satellite cells, suggesting the regulation of satellite cells by NGF or other neurotrophins.

The satellite cells retain their mitotic capability as revealed by their change in number after sciatic nerve injury (Pannese, 1964; Fenzi et al., 2001). Studies on axotomised neurons showed hypertrophy and hyperplasia of satellite cells after axotomy (Humbertson

et al., 1969), induction of satellite cell apoptosis both in neonates (Whiteside et al., 1998) and adults (Groves et al., 1997; McKay Hart et al., 2002), and the synthesis of neurotrophic factors, such as NGF and NT-3 (Zhou et al., 1999b). Under electron microscopy, Pannese et al. (2003) reported that the perineuronal satellite cells responded to axonal injury by forming new gap junctions between adjacent satellite cells, however no gap junctions were found between neurons and their surrounding satellite cells. Sensory neurons in injured ganglia may emit signals by mechanisms other than gap junctions to initiate the reaction of satellite cells after peripheral nerve injury (Aldskogius and Kozlova, 1998). However, the precise role of satellite cells in axotomised DRG is not clear.

1.3.6 Possible causes of axotomy-induced neuronal death

The exact mechanisms which mediate injury-induced changes in DRG neurons and the factors that ultimately determine whether a DRG neuron will survive or die after axotomy are poorly understood. One possible mechanism is the reduction in the normal retrograde transport of target-derived neurotrophic factors; this hypothesis is supported by the finding that blockade of normal axonal transport by vinblastine induces changes similar to transection with regard to neuropeptides, such as vasoactive intestinal peptide (VIP) and galanin (Knyihar-Csillik et al., 1991; Kashiba et al., 1992). Other signals are initiated or processed at the injury site and lead to the neuronal death or adaptive responses in DRG (Hanz et al., 2003). In addition, the expression of bcl-2 family in DRG neurons was regulated after sciatic transection, revealing that the ratio of two members of this family as cell death repressors, bcl-2 and bcl-$_X$L, to a cell death promotor Bax may be related to the susceptibility of DRG neurons to axotomy-induced cell death (Gillardon et al., 1996).

1.3.7 Injury-induced neuropathic pain

Neuropathic pain is induced by injury or disease of the nervous system. The pathophysiology of neuropathic pain is complex and remains poorly understood (Wang et al., 2002a). Following peripheral nerve injury, sympathetic axons which normally innervate blood vessels in DRG sprout to form varicose baskets around large diameter neurons (Chung et al., 1996; Zhou et al., 1996; Michael et al., 1997; Zhou et al., 1999b). This process has been implicated in the development and maintenance of neuropathic pain after nerve injury (Chung et al., 1996). The synthesis of NGF and NT-3 in reactive satellite cells and NGF and BDNF in activated Schwann cells may be involved in the induction of sprouting and neuropathic pain (Frostick et al., 1998; Zhou et al., 1999b). Delivery of exogenous NGF or BDNF directly into intact DRGs also triggers a persistent mechanical allodynia, but NT-3 does not (Zhou et al., 2000). Systemic injection of antisera to NGF, BDNF or NT-3 prevented sprouting and attenuated hyperalgesia and allodynia, confirming that endogenous NGF, BDNF and NT-3 induce sympathetic sprouting and are likely to be involved in the formation of neuropathic pain after peripheral nerve injury (Deng et al., 2000; Zhou et al., 2000).

Other events happening in spinal cord after axotomy are also linked to chronic neuropathic pain. In the normal animal, low threshold mechanoreceptors terminate in laminae III and IV and convey nociceptive inputs whereas unmyelinated C fibres, most of which are nocireceptors, terminate predominantly in lamina I and II in the dorsal horn of spinal cord. After peripheral axotomy, the central terminals of axotomised myelinated afferents, including Aβ fibres, were reported to sprout into lamina II with the consequence

of hyperalgesia (Woolf et al., 1992; Woolf et al., 1995; Bennett et al., 1996a; Doubell et al., 1997); these studies mainly used retrograde and transganglionic neuronal tracers to label A-fibres. However, recent studies demonstrated that the sprouting of A-fibres after axotomy is very limited (Bao et al., 2002; Hughes et al., 2003); a phenotypic change in small DRG neurons with an up-regulation of the binding site for tracers resulted in the misinterpretation of C-fibres to be A-fibres in previous studies. Whether the limited formation of aberrant sprouting in spinal cord could lead to neuropathic pain is unclear. In addition, the enhanced anterograde transport of BDNF to spinal cord following sciatic nerve injury (Michael et al., 1999) also contributes to the increased synaptic transmission and sensitises the pain pathway (Mannion et al., 1999; Thompson et al., 1999).

1.4 Changes of neurotrophin and neurotrophin receptor expression following axotomy

1.4.1 Axotomy-regulated neurotrophin expression

Disconnection of the sensory neurons from their peripheral targets by nerve transection, results in a decrease or cessation of the retrograde transport of target-derived neurotrophic factors, neuronal atrophy, neuronal loss and altered expression of neuropeptides and cytoskeletal proteins (Hoffman et al., 1987; Wong and Oblinger, 1987). Although the effects of axotomy are well known, their molecular mechanisms have been investigated by a few groups and many events remain uncharacterised.

To date, a few studies have examined the effect of peripheral axotomy upon messenger RNA (mRNA) and protein expression of neurotrophins and neurotrophin receptors in rat lumbar DRGs, using various semi-quantitative methods. These included *in situ* hybridisation, northern blotting, RNase protection assays and conventional RT-PCR, and the injury models used included spinal nerve transection, proximal and mid-thigh sciatic nerve transection and sciatic nerve crushing at various time points (Sebert and Shooter, 1993; Krekoski et al., 1996; Lee et al., 1998; Bergman et al., 1999; Michael et al., 1999; Shen et al., 1999a; Zhou et al., 1999b; Karchewski et al., 2002). Because some of the neurotrophic factors are synthesised in both DRG and target organs of sensory neurons, quantitation at protein and mRNA levels will provide a more thorough picture of the adaptive reaction of DRGs to peripheral axotomy.

In unoperated DRGs, it has been reported that most neurons, but not their satellite cells, express NGF mRNA (Zhou et al., 1999b), and around 40% of the lumbar DRG neurons are NGF-immunoreactive, especially small- to medium-sized ones (Lee et al., 1998). Peripheral axotomy, which produces an almost complete block of retrograde transport of NGF, produces an increase in NGF mRNA expression in the ipsilateral L4 and L5 DRGs from as early as 6 hours, to at least 3 weeks after the injury (Sebert and Shooter, 1993). One study showed that the NGF protein level, detected by enzyme-linked immunosorbent assay (ELISA), within the DRG decreased by 6 hours after tight ligation of spinal nerve, due to the effect of the decrease of retrograde transport, but almost fully recovered to normal levels by 2 days, suggesting the synthesis of NGF in DRG increased after peripheral nerve injury (Lee et al., 1998). The possible sources of newly synthesised NGF include satellite cells, which do not synthesise NGF in intact state, and sensory

neurons (Lee et al., 1998). This possibility is supported by previous reports which demonstrated an increased NGF mRNA expression in the axotomised DRG (Sebert and Shooter, 1993; Shen et al., 1997). NGF synthesis in many satellite cells occurs as early as 3 days after spinal or sciatic nerve ligation and is also increased in DRG neurons (Lee et al., 1998; Zhou et al., 1999b).

Sciatic nerve and spinal nerve transection leads to considerable increase in BDNF mRNA and protein in DRG throughout a period lasting from 12 hours to 4 weeks (Sebert and Shooter, 1993; Karchewski et al., 1999; Michael et al., 1999; Shen et al., 1999a). In uninjured DRG, BDNF is mainly synthesised in trkA-expressing small neurons exerting paracrine and autocrine functions (Michael et al., 1999; Karchewski et al., 2002); however, sciatic axotomy results in a major increase of BDNF mRNA from 2% of all neurons to 50% in trkB-expressing neurons and from 18% to 56% in trkC-expressing neurons without a significant change in the number of trkA-expressing neurons by 2 days (Michael et al., 1999). The increase in BDNF mRNA mainly occurs in the medium-to-large diameter DRG neurons (Cho et al., 1997; Tonra et al., 1998; Michael et al., 1999; Zhou et al., 1999a). Changes in mRNA level are reflected in BDNF protein expression with an increase of BDNF immunoreactivity in most neurons at 2 days and a shift to expression in mainly large neurons by 2 weeks (Karchewski et al., 2002). Expression of BDNF mRNA or protein in satellite cells has never been detected in intact or injured states.

In unoperated DRGs, NT-3 mRNA and protein are mainly localised in large neurons (Zhou et al., 1999b). After sciatic nerve transection at mid-thigh level, NT-3 mRNA increases in DRG by 75% at 16 hours after the lesion, and these levels persist for at

least 2 weeks (Zhou et al., 1999b). The increase of mRNA and protein is mainly localised to some satellite cells.

With regard to contralateral DRGs, although a few neurons appeared to be surrounded by NGF or NT-3-expressing satellite cells after axotomy, NGF and NT-3 mRNA did not change significantly in these ganglia after unilateral sciatic nerve lesion (Zhou et al., 1999b).

In addition to the up-regulation in DRGs, sciatic nerve transection also increases NGF mRNA in fibroblasts and Schwann cells of both proximal and distal stumps of the damaged nerve (Heumann et al., 1987; Lindholm et al., 1988). Schwann cells in the distal stump increased the synthesis of NGF, BDNF, NT-4 and $p75^{NTR}$ following sciatic nerve transection (Heumann et al., 1987; Meyer et al., 1992; Funakoshi et al., 1993).

1.4.2 Axotomy-regulated neurotrophin receptor expression

For quantitative analysis, radioisotope-labelled oligonucleotide probes have been used in most studies to determine the changes in the labelling density for mRNA encoding neurotrophin receptors (Verge et al., 1992; Krekoski et al., 1996; Shen et al., 1999b). The percentage and number of trkA-expressing neurons were studied in ipsilateral DRGs after spinal nerve ligation; a decrease in trkA mRNA was detected by ribonuclease protection assay as early as 3 days after surgery, reaching a maximum 30% decrease, and recovered to control levels by 2 months after injury (Shen et al., 1999b). Similar changes of trkA protein expression were observed after sciatic transection (Raivich et al., 1991; Verge et al., 1992; Krekoski et al., 1996; Shen et al., 1999b). TrkA protein is present in the satellite cells of

normal DRGs (Pannese and Procacci, 2002), but whether its expression in satellite cells is regulated by axotomy is not clear.

There are contrasting findings on the expression of $p75^{NTR}$: some studies observed a decrease in its expression (both mRNA and protein) after peripheral axotomy (Verge et al., 1992; Krekoski et al., 1996; Zhou et al., 1996; Bergman et al., 1999), whereas Ernfors et al. (1993) reported an increase of $p75^{NTR}$ mRNA one week after sciatic nerve crush using Northern blotting. However, sciatic nerve injury up-regulates $p75^{NTR}$ expression in satellite cells surrounding large neurons, from 1 week to 2 months afterwards (Zhou et al., 1996).

1.5 Gene expression pattern following axotomy: microarray studies

The adaptation of neurons to a certain stimulus or injury is first reflected at transcriptional and translational levels, and only subsequently structural and functional modifications. In addition to the studies focusing on individual genes, especially neurotrophins and their receptors, a few studies have been carried out using oligonucleotide or cDNA microarrays to investigate more regulated genes in the DRG after peripheral nerve injury (Fan et al., 2001; Costigan et al., 2002; Kubo et al., 2002; Xiao et al., 2002).

1.5.1 Introduction of microarray

Microarray is a new technology which can monitor the whole genome or thousands of selected genes on a single array and provide researchers with information on the complex interactions of a lot of genes simultaneously by examining their changes.

An array is an orderly arrangement of oligonucleotides or cDNA complementary to known and unknown genes on solid surfaces, generally on glass but sometimes on nylon substrates and made by high-speed robotics. There are two major applications for the microarray technology: (1) Determination of expression level (abundance) of genes; and (2) Identification of sequence (gene / gene mutation). There are two formats of microarray technology: (1) cDNA microarray. cDNA (500~5,000 bases long) is immobilised to a solid surface such as glass and exposed to a set of targets either separately or in a mixture. (2) oligonucleotide microarray. 20~80-mer oligonucleotide probes are synthesised and followed by immobilisation to a chip. These arrays are exposed to labelled sample RNA or DNA, hybridised, and the identity/abundance of complementary sequences are determined (http://www.affymetrix.com)(Shoemaker and Linsley, 2002). In the current study, Affymetrix Rat Neurobiology U34 array, which is an oligonucleotide array, was used to examine the mRNA expression of more than one thousand neurobiology-related genes.

1.5.2 Application of microarray to the study of peripheral nerve injury

A DRG contains the cell bodies of primary sensory neurons, satellite cells, Schwann cells, mast cells, fibroblasts, perineurial and endothelial cells; therefore microarray data show the pooled expression of genes in all of these cells. Commercial arrays and specific 'Custom-made' arrays, from which researchers can select the content of their arrays and the array size, have been used on various animal models of peripheral nerve injury to examine the gene expression changes over extended periods of time, ranging from 3 days to 4 weeks (Xiao et al., 2002), or focusing their observations only on one specific time point (Fan et al., 2001; Costigan et al., 2002; Kubo et al., 2002). In some studies, array hybridisations were

carried out in triplicate to reduce false positives and negatives, but, in some others, only one array per time point was performed. The alterations of gene expression in injured DRG are possibly triggered by a loss or decrease of trophic support from target organs or by other signals initiated or secreted at the injury site (as previously discussed). The differentially expressed genes appear to reflect the adaptive, maladaptive or compensatory responses, such as the capacity to survive the injury and regeneration of the injured axon after nerve transection, by structural and functional modifications of DRG cells (Xiao et al., 2002). Differences in mRNA levels, compared with controls, may result from changes in either the transcription rate or the half-life of the transcripts.

Comparison of the profiles of regulated genes between different studies, using different platforms to carry out array hybridisations, are complicated by the different criteria used to define the regulated genes, the different genes included in commercial and customised arrays, the various sciatic nerve injury models used and different time points chosen.

Recently Costigan M et al. (2002) published a study using 8.8K oligonucleotide arrays in triplicate to analyse genes regulated in the DRG three days following mid-thigh sciatic nerve transection. Of these, 52-54% of the genes included were detected in arrays and 240 (5.1%, including 2.8% up-regulated and 2.3% down-regulated) genes were regulated by axotomy, defined by the criteria >1.5-fold change and with a p value of <0.05, using unpaired, two-tail t-test (Costigan et al., 2002). 8.5K cDNA Incyte mouse GEM arrays were used in a study investigating the gene expression pattern in DRG 7 days after mid-thigh transection of sciatic nerve (Bonilla et al., 2002). Twelve genes were up-regulated by more than 2-fold and 4 genes down-regulated. Xiao et al. (2002) used 7.5K

cDNA arrays to analyse genes regulated in DRG 2, 7, 14 and 28 days following sciatic nerve transection at mid-thigh level with one array at each time point and observed 122 genes and 51 expressed sequence tags (ESTs) regulated at least at one time point after axotomy (Xiao et al., 2002). The genes up-regulated or down-regulated in injured DRG detected by microarrays were then classified into functional categories, including signal transduction, cytoskeleton, ion channel, cell surface receptors, growth factors, cytokines, neurotransmission and apoptosis, and analysed with the help of data mining tools (Kubo et al., 2002; Xiao et al., 2002).

However, microarrary hybridisation investigating the effects of exogenous neurotrophins (e.g. NT-3) on injured DRG at any time point after sciatic nerve transection has never been done. The time point of 2 weeks was chosen in this study to explore the molecular mechanisms which may be involved in my morphometric findings.

1.6 Effects of neurotrophins on sensory neurons following axotomy

After sciatic nerve injury, surviving DRG neurons undergo structural and biochemical changes including alterations in morphology and in expression of enzymes, peptides, growth-associated molecules, cytoskeletal proteins and receptors; many of these changes reverse after successful regeneration (Groves et al., 1996; Costigan et al., 2002; Xiao et al., 2002). Administration of exogenous trophic factors to the injured nerve may replace a missing component derived from the target organ.

1.6.1 Delivery of neurotrophic factors

For these neurotrophins to be of widespread use clinically, it is important that they can be applied via a less traumatic route such as via the systemic circulation rather than intrathecally or intraneurally as used by a number of groups so far. Application of neurotrophins to the proximal stump of transected nerve impedes the regeneration of nerve. Neurotrophins are proteins and cannot get access to the central nervous system through blood-brain barrier (BBB)(Poduslo and Curran, 1996; Pan et al., 1998). On the other hand, peripheral sensory neurons have no barrier in DRG to prevent systemic proteins from coming into contact and are less protected and are better candidates for neurotrophin therapy (Jacobs et al., 1976; Olsson, 1968). Intravenously (IV) administered NGF, and particularly NT-3 and BDNF, have been shown to accumulate in the endoneurial compartment, indicating that they are capable of reaching peripheral axons via an active transport mechanism (Poduslo and Curran, 1996). Furthermore, the capillaries of rat DRGs are fenestrated and will allow the passage of proteins into the extracellular space: horseradish peroxidase (Molecular weight (MW): 44,000) comes into contact with the perikaryal membranes of DRG neurons within 5 minutes of IV administration into the femoral vein (Jacobs et al., 1976). NGF administered subcutaneously by micro-osmotic pump reaches maximal serum concentrations 6 hours later, declining to a steady state level after 3 days (Tria et al., 1994); NT-3 (MW 15,000) has a higher permeability coefficient-surface area product than NGF at the blood-nerve barrier (Poduslo and Curran, 1996). These data indicate that NT-3 in the systemic circulation would come into contact with both the axonal and perikaryal membranes of DRG neurons, where trk receptors are expressed, very soon after pump implantation and start-up. Other chronic delivery systems include intrathecal delivery, subcutaneous or intraperitoneal injection, and targeted

administration to the nerve stump using bioresorbable materials, including fibrin glue, fibronectin mats and collagen. Fibrin glue is prepared from a mix of fibrinogen concentrate, aprotinin solution, thrombin and calcium chloride solution; the polymer network can slowly release the neurotrophic factor bound inside as the network is gradually lysed (Yin et al., 1998). However, direct application of trophic factors may be problematic when a long-term infusion is required. In future, gene therapy, transduced in adenovirus, or transfer of specific cells which produce the desired trophic factors may be applicable to peripheral nerve injury (Emerich et al., 1997; Hoffer and Olson, 1997).

1.6.2 Effects of NGF administration

The protective effect of exogenous NGF on axotomy-induced neuronal loss was demonstrated via intrathecal infusion (Ljungberg et al., 1999) and via proximal nerve stump (Otto et al., 1987; Rich et al., 1989) after either spinal nerve or sciatic nerve transection. In addition, after intrathecal, systemic and *in vitro* NGF treatment, the NGF-responsive neurons in unoperated lumbar DRGs up-regulate the expression of $p75^{NTR}$ and trkA receptor (Lindsay et al., 1990; Miller et al., 1991; Verge et al., 1992). The decrease of trkA and $p75^{NTR}$ mRNA DRG in both DRG and deafferented dorsal horn cells after peripheral nerve transection can be at least partly reversed by exogenous NGF (Fitzgerald et al., 1985; Verge et al., 1992). These observations suggest a positive feedback mechanism which may be important in the survival and differentiation of NGF-responsive neurons during development, and their maintenance and regeneration in the adult.

Furthermore, NGF can regulate levels of other neurotrophins; following systemic and intrathecal NGF injection, BDNF levels were increased in unoperated DRG by 24

hours, especially in trkA-expressing neurons (Apfel et al., 1996; Michael et al., 1997). Systemic NGF administration lasting for 3 days to 2 weeks after unilateral sciatic nerve transection results in up-regulation of BDNF mRNA in both the number and intensity in both neurons in injured and uninjured DRGs (Verge et al., 1996; Obata et al., 2003).

Either systemic, intrathecal, or local NGF administration has been reported to activate the existing signalling pathways in some DRG neurons and satellite cells, increasing the levels of phosphorylated-extracellular signal-regulated protein kinase-1/2 (p-Erk-1/2) in trkA-expressing small-to-medium neurons (Averill et al., 2001; Obata et al., 2003); the p-Erk-1/2 immunoreactivity was induced in medium-to-large neurons after peripheral nerve injury (Obata et al., 2003). In compartmented cultures of rat E14 DRG neurons, Watson et al. (2001) found that NGF and BDNF stimulated axon terminals and then activated Erk-5 pathway for retrograde signalling; at the same time, local Erk-1/2 signalling cascades were also activated, but restricted within the distal axon. In contrast, neurotrophin stimulation of cell bodies activates both Erk-1/2 and Erk-5 cascades (Watson et al., 2001). These may explain the discrepancies in studies using different routes of neurotrophin administration.

In addition to its effects on neurotrophins and their receptors, NGF supplied intrathecally or through the proximal stump of the nerve partly prevented or reversed the injury-induced degenerative atrophy, and the decreased expression of medium neurofilament subunit (NF-M), peptides α- and β-CGRP and substance P (Csillik et al., 1985; Fitzgerald et al., 1985; Rich et al., 1987; Verge et al., 1990; Inaishi et al., 1992; Verge et al., 1995). NGF administration also counteracts the injury-induced increases in immediate early gene protein cJUN (Gold et al., 1993), peptides galanin, neuropeptide Y

(NPY), VIP and cholecystokinin (CCK)(Verge et al., 1995). Daily injection of NGF antiserum to normal adult rats results in a decreased axonal calibre and neurofilament expression in the proximal axon of DRG neurons, implying the importance of retrogradely transported NGF in maintaining the axonal calibre, at least in certain subsets of neurons (Gold et al., 1991). Functionally, NGF application to the transected proximal nerve stump counteracts the decrease in primary afferent depolarization after sciatic nerve axotomy (Fitzgerald et al., 1985).

Importantly, subcutaneous injections of NGF caused mechanical and heat hyperalgesia and an increase in nociceptive fibre sprouting (Lewin and Mendell, 1993), which may be a problem in the event of therapeutic use of NGF. Phase I, II and III clinical trials of recombinant human NGF (rhNGF) for treating diabetic peripheral neuropathy have revealed significant beneficial effects after 6 months of treatment with injection site hyperalgesia the most common adverse event (Petty et al., 1994; Apfel et al., 1998).

1.6.3 Effects of BDNF administration

Studies of the effects of BDNF on DRG after peripheral nerve injury are limited. Intrathecal infusion of BDNF has no rescue effect on neuronal loss of T13 DRG after spinal nerve transection (Ljungberg et al., 1999); however, systemic BDNF administration improves the diameter and myelin thickness of regenerating axons after sciatic nerve transection and repair and increases functional recovery by 80 days (Lewin et al., 1997).

1.6.4 Effects of NT-3 administration

Exogenous NT-3 has been shown to have beneficial effects on morphological changes, gene expression alterations, electrophysiological deficit and functional recovery after peripheral nerve injury (Verge et al., 1996), although its mechanisms of action are not clear. NT-3 administration intrathecally or at the proximal stump effectively prevented lumbar (Groves et al., 1999) and thoracic (Ljungberg et al., 1999) DRG neuron loss after peripheral nerve transection. Intrathecal administration of NT-3 also promotes axonal regeneration across the dorsal root entry zone (DREZ) which is a CNS-PNS interface in spinal cord, and recovery of proprioceptive function after dorsal rhizotomy (Ramer et al., 2002). Intrathecal NT-3 administration for one week was shown to partly counteract the decreased $p75^{NTR}$ expression in injured DRG after 2 week axotomy (Verge et al., 1996). NT-3 administration, either to the proximal nerve stump or systemically, can partly prevent the decrease of conduction velocities and excitatory postsynaptic potentials (EPSPs) after axotomy or in streptozocin (STZ)-induced diabetic rats (Munson et al., 1997; Mendell et al., 1999). Subcutaneous administration of NT-3 also reduced functional and electrophysiological deficits of large-fibre sensory neuropathy induced by vitamin B_6 (Pyridoxine)(Helgren et al., 1997). Delivered by grafting impregnated fibronectin in the axotomised sciatic nerve, NT-3 does not effect the expression of CGRP, SP or VIP, but attenuates the up-regulation of NP-Y at 30 days after axotomy (Sterne et al., 1998). While NT-3 prevents neuron loss (Ljungberg et al., 1999), it did not prevent apoptosis of DRG neurons *in vitro* (Edstrom et al., 1996). Whether the neuron number, neuronal apoptosis, expression of neurotrophins, neurotrophin receptors and neurofilaments, and the gene expression profile in axotomised DRG are regulated by systemic NT-3 administration *in vivo* is not clear.

1.6.5 Other neurotrophins/cytokines

Cytokines are a group of polypeptide mediators, and include interleukins, chemokines, tumour necrosis factors, interferons, colony stimulating factors, growth factors, neuropoietins and neurotrophins. Glial-derived neurotrophic factor (GDNF) (Lin et al., 1993), ciliary neurotrophic factor (CNTF) (Sendtner et al., 1994), basic fibroblast growth factor (bFGF)(Eckenstein, 1994) and leukemia inhibitory factor (LIF)(Murphy et al., 1993) support the survival of particular sensory neurons in culture. *In vitro* studies using DRG explants showed that GDNF stimulates axonal outgrowth of IB4-labelled small diameter neurons which do not express trkA or other neurotrophin receptors (Leclere et al., 1998; Bennett et al., 1998). GDNF also reverses the decrease in IB4 labelling in DRG neurons, improves the conduction velocity of these small diameter sensory neurons after axotomy (Bennett et al., 1998), and also reduces apoptosis in explants (Leclere et al., 1998).

1.7 Neurogenesis in nervous system

1.7.1 Neurogenesis in adult central nervous system

There is thought to be very limited neuronal generation/regeneration in post-natal CNS. Exceptions to this generalisation are two areas of the post-natal brain in mammals which are known to generate new neurons: the dentate gyrus of the hippocampal formation, and the subventricular zone (SVZ) which has a projection through the rostral migratory stream to the olfactory bulb (Eriksson et al., 1998; Ciaroni et al., 1999; Doetsch et al., 1999). Proliferating SVZ cells are located along the lateral edge of the lateral ventricle,

mainly along the frontal horn. Olfactory receptor neurons are ageing and dying and being replaced continuously by new neurons generated from a pool of neuronal progenitor cells. Neurogenesis in these regions mainly depends on proliferation of stem cells or undifferentiated precursor cells (Cameron et al., 1993; Ciaroni et al., 1999), which can be identified by ^{3}H-thymidine and BrdU incorporation.

Neural stem cells (NSCs) are defined as self-renewing multipotent cells that have the potential for bidirectional glial/neuronal differentiation into neurons, astrocytes and oligodendrocytes in the developing CNS (Reynolds and Weiss, 1992). NSCs have been shown to exist throughout life in the adult brain, particularly the subventricular zone and the dentate gyrus of the hippocampus (Reynolds and Weiss, 1992; Richards et al., 1992). Stem cells can also be isolated from other regions of brain and spinal cord (see Gage et al, 1995; Gage, 2002; Shihabuddin et al., 2000). In addition, marrow stromal cells (MSCs) can differentiate into neurons and astrocytes after exposure to a brain environment *in vivo*, or to epidermal growth factor (EGF) and BDNF *in vitro* (Eglitis and Mezey, 1997; Brazelton et al., 2000; Woodbury et al., 2000). Transplantation studies in rodents have showed that NSCs from embryonic hippocampus could bring a degree of recovery of cognitive and neuromotor function after brain insults, but the structural basis for this repair is not clear yet (Philips et al., 2001; Veizovic et al., 2001).

In the rodent brain, neurogenesis, assessed using BrdU incorporation, starts to increase at 7 days and peaks at 11 days after brain ischaemia (Sharp et al., 2002), which may explain the partial recovery of function following global brain ischaemia. Many factors are able to regulate neural stem cells *in vitro*, including bFGF, epidermal growth factor (EGF), transforming growth factor (TGF), insulin-like growth factor-1 (IGF-1),

glutamate, γ-aminobutyric acid (GABA), opioid peptides, platelet-derived growth factor (PDGF), CNTF and neurotrophins (Kuhn et al., 1997; Cameron et al., 1998; Namaka et al., 2001).

Nestin, a member of intermediate filament protein family, is expressed in common progenitors or neural stem cells which can give rise to both neurons and non-neuronal cells (Messam et al., 2000). In mice, it is expressed early from embryonic day E7.5 in many proliferating CNS regions, followed by expression in the rostral and caudal neuronal tube at E10.5, cerebellum at E15.5, and ventricular and subventricular areas of the developing telencephalon at postnatal day P0 (Dahlstrand et al., 1995). Nestin expression declines rapidly during the cells transition from proliferating to postmitotic state, and this is accompanied by the onset of expression of glial fibrillary acidic protein (GFAP) in astrocytes and neurofilaments in neurons (Dahlstrand et al., 1995). In rat brain, nestin expression diminishes by P6 in the spinal cord and by P21 in the cerebellum (Hockfield and McKay, 1985). Nestin is also expressed in endothelial cells and ependymal cells in the subventricular zone in CNS and in Schwann cells in PNS (Friedman et al., 1990; Tohyama et al., 1992; Duggal et al., 1997). Nestin has been detected in some pathological conditions including CNS tumours, where its amount has been correlated with the severity of the tumour (Dahlstrand et al., 1992), and excitotoxic and traumatic brain injury. After focal brain ischemia and spinal cord injury, nestin expression is present in some cortical neurons and reactive astrocytes (Clarke et al., 1994; Frisen et al., 1995; Duggal et al., 1997). The re-expression of this early fetal gene and co-expression of a few markers for early embryonic development and neuronal differentiation, such as vimentin, embryonic neural cell adhesion molecule (E-NCAM), neuron-specific enolase (NSE) and microtubule-associated

protein 2 (MAP-2)(Vinores et al., 1984; Geisert et al., 1990; Le Gal La Salle et al., 1992), in response to cellular stress in the CNS may represent a reversion to an more immature, embryonic cell state and a potential to repair damaged neuronal processes and synaptic plasticity (Duggal et al., 1997), or it may indicate the recent formation of those neurons from nestin-IR precursors (Woodbury et al., 2000).

1.7.2 Neurogenesis in adult peripheral nervous system

During development, DRG neurons are derived from precursors in the neural crest cells and neurogenesis was proposed to end perinatally in the PNS (Farinas et al., 1996; Greenwood et al., 1999). Recent work has suggested that neuronal differentiation continues postnatally (Farel, 2002). In rat DRG, the first evidence for the existence of possible post-natal neurogenesis or continuing neurogenesis was based on the slow accretion of neuron numbers occurring in adulthood (Devor and Govrin-Lippmann, 1985). In that study, a profile-based neuron counting method was applied to count neurons in L4/L5 DRGs, but the researchers did not try to observe dying neurons. Using profile counts or stereological counting methods to support the age-related increase in DRG neurons, postnatal neurogenesis in rat DRG has also been observed by several authors (Cecchini et al., 1995; Popken and Farel, 1997; Farel, 2002, 2003); however, whether neurogenesis occurs in DRGs during adult life is still unclear. In contrast, others reported that neuron number remains unchanged after birth (Pover et al., 1994; Mohammed and Santer, 2001). Profile-based neuron counts are potentially biased by changes in neuron size, such as occurs in male rats with age. Stereological counting techniques make less assumptions about size and shape and so are less likely to be biased by any such changes (see Coggeshall, 1992).The

lack of observation of dying neurons also makes any conclusions about neuronal death very suspect. *In vitro*, neurogenesis was seen in DRG cell culture from postnatal (day 1-2) mice in the presence of bFGF, epidermal growth factor, NGF, BDNF, NT-3 and GDNF (Namaka et al., 2001). Some neuron-like cells in these newly generated spherical cell aggregates appear to incorporate thymidine analogue BrdU which detects DNA synthesis, and these cells are labelled by neuron-specific markers neurofilament-160 and microtubule-associated protein-2 (MAP-2), and extended axon-like processes and presented electrical properties characteristic of neurons. Using another experimental model, in which deficiency of vitamin E enhances neurogenesis in a specific period of time, some satellite cells and round, spindle or bell-shaped cells were labelled by BrdU (Ciaroni et al., 2000). Double stained by BrdU and NF-200 antibody, some of these cells were suggested to be transitional neuroblasts which exist in developing DRGs. Whether nerve transection leads to neurogenesis in PNS and which are the factors regulating any neurogenesis in PNS are not well studied.

In this thesis, the hypotheses are:

(1) Axotomy may cause certain cells to differentiate into DRG neurons and NT-3 may stimulate this process.

(2) Axotomy and systemic NT-3 administration may produce molecular changes in DRG that may assist regeneration.

CHAPTER 2: MATERIALS AND METHODS

2.1 Animals

A total of 150 adult male Sprague-Dawley rats weighing between 275 – 325 g were used. Animals were housed singly with food and water available ad libitum, and they were kept in a colony room maintained at 22°C and with a 12-h alternating light-dark cycle. Experiments conformed to Home Office regulations.2.0.1 Surgical procedures: anaesthesia, analgesia and post-operative care.

Animals were anaesthetised with halothane via a Boyle's inhalational apparatus. An intramuscular injection of Temgesic (buprenorphine hydrochloride: Reckitt and Colman), 10 μg/kg, was injected intramuscularly for post-operative analgesia.

All animals were monitored daily for signs of autotomy after procedures. Two rats were found to have more than two toes missing and were humanely destroyed.

Table 2.1: Numbers of animals used and the surgical procedures carried out

Procedure	Number of animals
Right sciatic nerve transection	13
Right sciatic nerve transection + vehicle administration	48
Right sciatic nerve transection + NT-3 administration	52
Unoperated	45
Total	158

2.1.1 Laboratory reagents

General lab chemicals were from Sigma and of analytical grade. Unless stated otherwise, solutions and buffers were prepared using de-ionised water. Autoclaving procedures were carried out at 121 °C, 15 lbs/square inch for 30 minutes.

2.1.2 Surgical procedures: anaesthesia, analgesia and post-operative care

Under halothane anaesthesia, an 1 cm incision was made on the skin at mid-thigh level and the right sciatic nerve was exposed after carefully blunt dissecting the muscles surrounding it. The nerve was tightly ligated at mid-thigh level by sterile silk suture material, and transected at 2 mm distal to the tie. A 5mm length of the distal stump was removed to prevent any regeneration, the muscle was sutured in layers. After application of antibiotic powder (Cicatrin), the skin incision was closed by surgical stapler. Another incision was then made in the skin of the dorsum of the rat, where it cannot be reached by claws or mouth, and a subcutaneous pocket was created by separating the dermis from the underlying muscle layer. An "Alzet" micro-osmotic pump (Alza Corporation, USA) containing a solution of human recombinant NT-3 in vehicle (Regeneron Pharmaceuticals Inc.) or vehicle (4.5% mannitol, 0.5% sucrose and 10mM histidine, pH 5.0 in sterile saline) in the operated control group animal was inserted into the pocket and the incision sutured. Treatments lasted for 1 week (pump model 2001, 100μl 12.5mg/ml NT-3), 2 weeks (model 2002, 200μl 3.125 or 12.5mg/ml NT-3), or 4 weeks (model 2004, 200μl 6.25 or 25mg/ml NT-3). For the 1 day (24 hours) administration group, one subcutaneous injection of 0.179mg/15μl NT-3 or 15μl vehicle was given immediately after the operation. Antiseptic "Sprilon" spray was then applied to the wound and the animals allowed to recover.

Seven groups of animals were used for the morphometric and neuron apoptosis study: (1) unoperated rats (n=7); (2) 2 weeks after axotomy with vehicle pump infusion (n=4); (3) 2 weeks after axotomy with NT-3 (0.625mg) pump infusion (n=5, 0.045mg/day); (4) 4 weeks after axotomy without any treatment (n=5); (5) 4 weeks after axotomy with

vehicle pump infusion (n=5); (6) 4 weeks after axotomy with NT-3 (1.25mg) pump infusion (n=5, 0.045mg/day); (7) 4 weeks after axotomy with NT-3 (5mg) pump infusion (n=4, 0.179mg/day).

Nine groups of animals were used for the real-time quantitative PCR investigation: (1) unoperated rats (n=5); (2) 1 day after axotomy with subcutaneous 15μl vehicle injection (n=5); (3) 1day after axotomy with subcutaneous 0.179mg/15μl NT-3 injection (n=5); (4) 1 week after axotomy with vehicle pump infusion (n=5); (5) 1 week after axotomy with NT-3 (1.25mg) pump infusion (n=5, 0.179mg/day); (6) 2 weeks after axotomy with vehicle pump infusion (n=5); (7) 2 weeks after axotomy with NT-3 (2.5mg) pump infusion (n=5, 0.179mg/day); (8) 4 weeks after axotomy with vehicle pump infusion (n=5); (9) 4 weeks after axotomy with NT-3 (5mg) pump infusion (n=5, 0.179mg/day).

Three groups of animals were used for microarray analysis: (1) unoperated rats (n=12); (2) 2 weeks after axotomy with vehicle pump infusion (n=12); (3) 2 weeks after axotomy with NT-3 (2.5mg/2 weeks = 0.179mg/day) pump infusion (n=12)

2.1.3 Preparation of micro-osmotic pump with NT-3 and vehicle solution.

Alzet micro-osmotic pumps (Alza co., Palo Alto, CA), model 2001 (100 μl, for 1 week administration), 2002 (200 μl, for 2 weeks) and 2004 (200 μl, for 4 weeks), were used in the study for delivering NT-3 and vehicle into the subcutaneous space, and proper sterile technique was used during the preparation of solution and filling of the pumps. The subcutaneous micro-osmotic pumps we used to deliver NT-3 to the systemic circulation for up to 4 weeks after sciatic nerve transection avoid fluctuating serum NT-3 levels produced by repeated injections or weekly refilling of reservoirs. NT-3 (18.8mg/vial) was kindly

provided by Regeneron Pharmaceuticals, Inc., aliquotted and stored at -70° C. Five dilutions of NT-3 were prepared for morphometric and molecular studies using sterile normal saline: 0.18 mg/15µl (1 day, molecular study), 1.25 mg/100 µl (1 week, molecular study), 0.625 mg/200 µl (2 week, morphometric study), 2.5mg/200µl (2 week, molecular study), 1.25 mg/200 µl (4 week, morphometric study) and 5 mg/200 µl (4 week morphometric and molecular studies).

2.2 Axotomy-induced apoptosis in adult rat dorsal root ganglia

2.2.1 Tissue preparation

Two and four weeks after the procedure, the rats for morphometric study were deeply anaesthetised with intraperitoneal pentobarbitone (60mg/kg) injection and perfused transcardially with 600ml of 4% depolymerised paraformaldehyde in 0.1M phosphate buffer, pH 7.4 over a period of 15 minutes at room temperature. The solutions were pumped into the aorta at a pressure of approximately 13 lb/inch^2 by means of a catheter connected to a peristaltic pump. The left and right L4 and L5 DRGs, which appeared as yellow swellings, were removed with 2 mm of roots and nerve either side and allowed to fix in the same fixative for 24 hours. The DRGs were then held between two biopsy sponges, put in "Tissue-tec" cassettes and processed overnight by a Shandon Citadel automated tissue processor as follows:

(1) Formol alcohol: 1hr. 15 mins; (2) 70% alcohol: 1 hr; (3) 90% alcohol: 1 hr; (4) Absolute alcohol 1: 1 hr; (5) Absolute alcohol 2: 1hr 30 mins; (6) Absolute alcohol 3: 1hr.

30 mins; (7) Chloroform 1: 1 hr. 30 mins; (8) Chloroform 2: 2hrs; (9) Molten paraffin wax 1: 1 hr; (10) Molten paraffin wax 2: 1 hr; (11) Molten paraffin wax 3: 1 hr. 30 mins.

Serial 3 μm sections of each ganglion were cut longitudinally and mounted onto glass slides in strips of 5. All sections were retained.

2.2.2 Apoptotic neuron counting

Every other slides prepared from perfused animals were deparaffinized, rehydrated and stained with acidified 0.01% cresyl fast violet at 60°C for 20 minutes before being dehydrated, cleared and mounted. Each section was then examined at 250 × magnification for the presence of apoptotic neurons using morphological criteria, including condensed nuclear chromatin, irregular nuclear and cellular shape and darkly staining cytoplasm. The incidence of apoptotic neurons was expressed as a fraction of half of the estimated total number of neurons in the ipsilateral L4 and L5 DRGs (pooled). Structures 15-30μm in diameter that were empty except for the remains of some condensed DNA, and which were surrounded by satellite cells corresponded to the "ghost cells" reported from cell culture studies of sympathetic neurons undergoing apoptosis (Edwards and Tolkovsky, 1994). These were not counted as neuronal, apoptotic or otherwise.

2.2.3 Neuronal counting

To examine if there was neuronal loss two and four weeks after axotomy and systemic NT-3 administration, neuronal counting was carried out on the same stained 3 μm cresyl fast violet sections which we used to identify apoptotic neurons. A physical disector method of stereology was used (Sterio, 1984; Coggeshall, 1992; Groves et al., 1999). Ten

pairs of sections, regularly spaced through DRG, were examined in each ganglion, therefore the total number of sections containing neuronal cell bodies were divided by 10 (= x) to find the number of sections in an interval between levels. The starting pair was chosen by selecting a number randomly between 1 and x. The first section in each pair of sections is called the "reference section": the next section is called the "look-up" section. Section pairs were examined at a magnification of 250× on a Zeiss microscope with planapochromat objectives, connected to a Leica Quantimet 500 image analyser, which consists of a JVC 3CCD video camera connected to an IBM-PC Pentium with associated video processing hardware and Leica image analysis software. The magnification obtained with the image on the monitor is around ×1000 with the ×25 objective. The image analyser and video camera were used to store the image of the field of reference section being examined and we could examine the contiguous look-up section to count the particles at the same time. Only the nuclei which existed in the reference section but not in the look-up section (called "tops") were counted (**Figure 2.1**). More than 100 "tops" should be counted for an accurate estimation of nuclear density, and it has been concluded that more than 200 "tops" counted will not lead to any greater accuracy (Sterio, 1984; Gundersen et al., 1988). The areas of partial disectors were measured using the image analyser. The mean section thickness of the reference sections was measured using a Heidenhain MT12 microcator attached to the stage of a microscope in conjunction with a ×63 oil immersion objective with a numerical aperture of 1.4, giving a depth of field of around 0.5μm. The thickness of a section was determined by establishing the top and bottom of the section, and the thickness could be read from the microcator. The mean ganglion area of the reference section sampled was calculated and multiplied by the average thickness of the sections and

the total number of sections to obtain the volume of the ganglion (V_{ref}). By dividing the number of tops counted for each ganglion by the product of the mean reference section thickness and the total areas of the disectors, the numerical density of neuronal nuclei can be calculated. The total number of neurons was then estimated by multiplying the ganglion volume (V_{ref}) by the numerical density. If enough tops are sampled in an unbiased way, the numerical density will reflect the density of neurons in the ganglion examined.

Numerical density = $\Sigma Q^{-} \div \Sigma V_{disectors}$

(Q^{-} = number of tops in each disector; $V_{disectors}$ = volume of each disector; Σ: the sum of a set of values)

The volume of the ganglion was estimated using the Cavalieri principle (Gundersen et al., 1988)

Volume of ganglion (V_{ref}) = (mean reference section area) × (mean section thickness) × (number of sections)

The degree of neuronal loss for each animal was calculated as the ratio of the number of neurons in the ipsilateral (right) to that in the contralateral (left) L4 and L5 DRGs. A value of 1 for this ratio would indicate equal numbers of neurons in the ipsi- and contralateral ganglia, and a value lower than 1 indicates fewer neurons in the injured DRGs than in the contralateral ganglia, as no degenerating neurons were seen in any serially-sectioned contralateral or control DRGs.

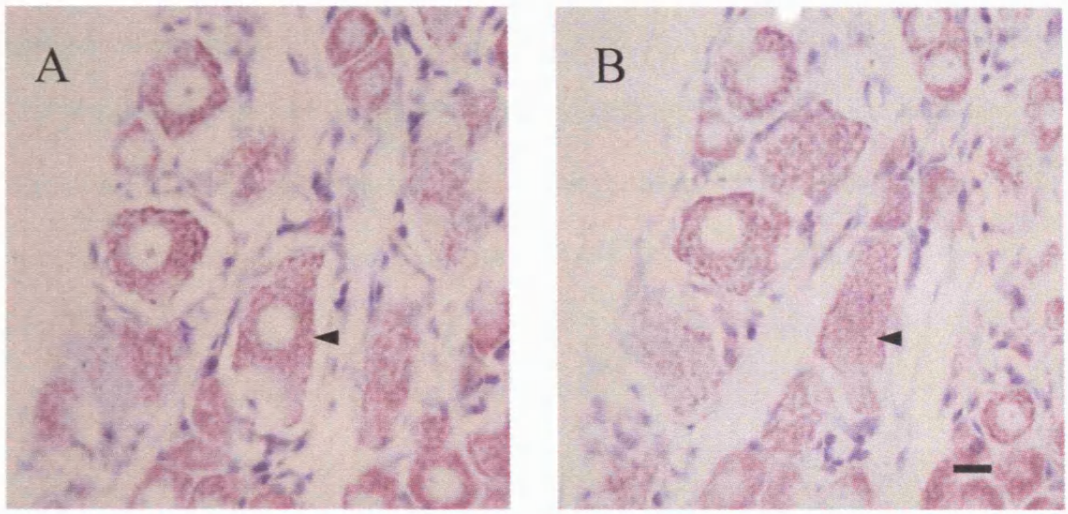


Figure 2.1: Examples of a reference section (A) and an adjacent "look-up" section (B). The arrowhead indicates a neuronal nucleus that appears in the reference section but not in the look-up section, and is therefore counted. Scale bar=10 μm.

2.2.4 Measurement of neuron diameters.

The diameters of sensory neurons in L4 and L5 DRGs were measured using a Leica Quantimet 500 image analyzer and JVC 3-CCD colour video camera attached to a Zeiss microscope. Three sections were taken from each ganglion and neuronal areas were measured if the nucleus was bound by a defined nuclear membrane and contained at least one nucleolus. A total of more than 600 neurons were examined in each side and three animals were studied in group (i)-(iii) and (v)-(vii) for morphometric study. The actual diameters of these neurons were converted from their calculated area and plotted in histogram form. Cell size in sections of paraffin-embedded ganglia are reported to be reduced compared with those in frozen sections (McCarthy and Lawson, 1990), but the pattern of neuronal size distribution are unaffected (Lawson and Harper, 1985). For minimising the influences resulted from tissue preparation, including transcardial perfusion with 4% depolymerized paraformaldehyde and processing in a Shandon Citadel automated

tissue processor, the graph of results was shown by comparisons between both side L4+L5 DRGs in each animal.

In this study, the pattern of cell diameter distribution was taken into consideration instead of actual size; the latter is only useful if all the ganglia were from animals of the same age and processed at the same time, and should be assessed using stereological criteria.

2.3 Immunohistochemical study of activated caspase-3, nestin, β-III tubulin, trkA, trkC and CGRP

2.3.1 Immunohistochemistry for activated caspase-3 and nestin

Half of the remaining serial 3μm sections from the study of apoptotic neuron counting (section 2.2.2) were immunostained for activated caspase-3, a proteolytic enzyme that is activated during apoptosis. Three 3μm sections from 3 non-adjacent levels, equally spaced through DRG, in unoperated (n=4), 4 week axotomy + systemic vehicle administration (n=4), 4 week axotomy + systemic NT-3 (5mg) administration (n=4), and 8 week axotomy + systemic vehicle administration (n=4) rats were used for nestin immunohistochemistry. Remaining sections were used for double-labelling experiments.

Sections were deparaffinized in xylene and rehydrated in graded alcohols before being immersed in 600ml of 0.1M citrate buffer pH 6.0 and microwaved at full power for 20 minutes in a 650 watt microwave oven. The slides were then washed in 3 changes of phosphate-buffered saline pH 7.4 (PBS) and blocked in 10% non-immune swine serum for

30 minutes. This was tipped off before the sections were incubated overnight at room temperature in either rabbit polyclonal anti-activated caspase-3 antibody (R&D Systems Europe Ltd) diluted 1:1000 in PBS containing 0.1% triton-X (PBS-TX) or mouse monoclonal anti-nestin antibody (1:1000; RAT-401: DSHB). Sections of E14 rat embryos were used as positive controls.

After washing in PBS for 3 times (5 minutes each wash), the sections were incubated for 1 hour at room temperature in either biotinylated swine anti-rabit secondary antibody (1:500 in PBS-TX; Dako) or biotinylated goat anti-mouse secondary antibody (1:200 in PBS-TX; Dako). All sections were then rinsed 3 times in PBS and further incubated for 1 hour at room temperature in peroxidase-conjugated strptavidin (1:300 in PBS-TX: Sigma). All sections were then immersed in 0.05% diaminobenzidine (DAB) in PBS containing 0.04% nickel chloride ($NiCl_2$) to intensify the chromogen reaction and 0.01% hydrogen peroxide (H_2O_2) for 10 minutes to visualise antibody binding. The sections were then washed in PBS twice, rinsed in distilled water, dehydrated through graded alcohols and mounted.

Immunoreactive cells and neurons appear blue/black. The diameters of nestin-immunoreactive neurons were measured using the methods described in section 2.2.4. The incidence of nestin-immunoreactive DRG neurons was estimated by counting the number of nestin-immunoreactive and non-nestin-immunoreactive neurons at 3 widely spaced levels from each ganglion; all profiles with nuclei were counted. Nestin-immunoreactive and non-nestin-immunoreactive neurons had to be at least 15μm in diameter, and to have the morphology of neurons (round or polygonal shape, large nuclei with nucleoli and little chromatin) to be counted.

2.3.2 Double immunofluorescence for nestin and β-III tubulin, trkA, trkC or CGRP

Double-labelling immunofluorescence was used to investigate the co-expression of nestin (mouse monoclonal antibody) with the neuronal antigen β-III tubulin (1:1000, mouse monoclonal; Sigma), trkA (1:500, rabbit polyclonal; Santa Cruz), trkC (1:100, rabbit polyclonal; Santa Cruz), or CGRP (1:1000, rabbit polyclonal; kindly provided by P. Facer, Imperial College London).

For polyclonal antibodies, the sections were deparaffinized, rehydrated, immunostained for nestin in the same way as described in 2.3.1 except the fluoroscein-conjugated avidin was used instead of horseradish peroxidase-conjugated streptavidin. Polyclonal antibodies were diluted in PBS-TX and were incubated with the nestin primary antibody and visualised with Texas red-conjugated goat anti-rabbit secondary antibody (1:100; Sigma). After washing in PBS the sections were coverslipped in Citifluor (Agar) and viewed with a Zeiss Axioskop fluorescence microscope with filter sets for fluoroscein and Texas red.

For the monoclonal anti-β-III tubulin antibody, nestin was immunostained first, incubated with biotinylated secondary antibody and then fluoroscein-conjugated avidin. The slides were then blocked in rabbit anti-mouse antibody (1:10 in PBS-TX) for 30 minutes before incubation in the second primary antibody and subsequent detection with a Texas red-conjugated goat anti-mouse secondary antibody (1:100 in PBS-TX; Sigma). After washing in PBS, these slides were coverslipped and viewed under fluorescence microscope as described in previous paragraph.

Controls for double labelling included omission of primary or secondary antisera and reversal of the order of primary antibody staining.

2.4 mRNA expression: studied by real-time quantitative PCR

2.4.1 Total RNA extraction

At 1 day, 1 week, 2 weeks and 4 weeks after surgical procedure, 10 rats (5 vehicle-treated and 5 NT-3-treated), at each time point were terminally anaesthetised with an intra-peritoneal injection of pentobabitone (60 mg/kg). Five additional unoperated animals provided normal tissue. The right and left L4 and L5 DRGs were identified by dissection of their spinal nerves as far as they fuse to form the sciatic nerve, and were removed rapidly and kept separate in ice cold 150 µl RNAlater (Qiagen) to minimise RNA degradation. For minimising the interference of RNA contributed by cells other than DRG cells, the surrounding capsule tissue was stripped off by fine surgical forceps under operative microscope. The protocol of RNeasy Mini Kit from Qiagen was used to isolate total RNA. A 2 ml tube was used to prepare the lysis Buffer RLT (add 6 µl β-Mercaptoethanol to 600 µl Buffer RLT from RNeasy Mini Kit). These ganglia were then cut thoroughly in appropriate amount of Buffer RLT using sterile disposable scalpels, and the L4 and L5 DRGs on the same side in each animal were pooled together and processed as follows: the cut-up DRGs were pipetted into a 2 ml tube with Buffer RLT in it, and homogenised by centrifuging in QIAshredder spin columns at 14,000 rpm (11,000 × g) for 2 minutes at RT. The used column was discarded, replaced by a cap, and the collection tube was centrifuged

at the same speed for another 3 minutes. The supernatant was mixed to 600 µl 70% ethanol in a new 2 ml microcentrifuge tube, and aliquots of 700 µl were loaded and centrifuged at 10,000 rpm (8,000 × g) for 15 seconds in an RNeasy mini column to isolate total RNA. 350 µl Buffer RW1 was added to the column and centrifuged at 10,000 rpm for 15 seconds to wash the RNA bound to the membrane. In order to prepare pure total RNA without DNA contamination, DNase Set (Qiagen) was used as described below.

10 µl DNase I solution (30 units) was gently mixed with 70 µl Buffer RDD and pipetted directly onto the RNeasy mini column for incubation for 20 mins at 20-30 °C. 350 µl Buffer RW1 was applied into the RNeasy mini column and centrifuged at at 10,000 rpm for 1 minute to clear up the membrane-bound RNA. The RNeasy column was then transferred into a new 2 ml RNase-free collection tube. 500 µl of Buffer RPE was pippetted onto the RNeasy column, followed by centrifuging at 10,000 rpm for 15 seconds. The RNeasy column was transferred into a new 2ml RNase-free collection tube and was centrifuged at full speed for 1 min. RNeasy column was transferred to a 1.5ml RNase-free collection tube and eluted by 50 µl RNase-free water which was applied directly onto the RNeasy silica-gel membrane and stayed for 1 minute before being centrifuged at 10,000 rpm for 1 minute. The extracted total RNA was proceeded to the next step for precipitation.

2.4.2 Precipitation of total RNA

A 1/10 volume of 2M Sodium acetate pH 4.0 was added to an RNA sample resulting from RNeasy mini kit extraction procedure. One volume of Isopropanol was added and vortexed briefly. The mixture was incubated overnight at –20 °C to precipitate the RNA. The RNA was pelleted by centrifuging at 12,500 rpm (10,000 × g) for 15 minutes

at 4 °C. The supernatant was decanted and the pellet was washed with 180 µl of ice cold 75% ethanol to remove the salt and centrifuged at 12,500 rpm (10,000 × g) for 15 minutes at 4 °C. The supernatant was drained and the tube was left drying in the air for 5 minutes. The RNA was resuspended in appropriate amount of RNA-free water to the concentration of 2 µg total RNA in 12.5 µl water.

2.4.3 Quantitation of total RNA in solution

Concentration and purity of total RNA were determined by measuring the ratio of the absorbance of RNA samples at 260nm and 280 nm respectively (ratios between 1.8 and 2.0 are acceptable). 2 µl of RNA sample was diluted into 78 µl of dH_2O. RNA samples were measured in cuvettes in a GeneQuant II spectrophotometer (Pharmacia Biotech).

2.4.4 Reverse transcription

Ominiscript RT Kit (Qiagen), which is suitable for 50 ng –2 µg total RNA, was used for the reverse transcription reaction for cDNA (complementary or copy DNA) library synthesis. All the reagents were thawed on ice and the following procedures were also carried out on ice. Oligo-dT primer was used to anneal to the 3' polyadenyl "tail" of the mRNA. A 20 µl master mix was prepared by adding 12.5 µl template total RNA, 2 µl 10 × Buffer RT, 2 µl dNTP (5 mM), 2 µl oligo-dT primer (10 µM), 0.5 µl RNase inhibitor and 1 µl Omniscript reverse transcriptase (company Roche, 10 units/µl) into thin-walled PCR tubes and mixed thoroughly. The reaction was carried out in a in a GeneAmp PCR system

9700 (Applied Biosystems). The 3-step programme included incubation at 37 °C for one hour, reverse transcriptase inactivation at 93 °C for 5 mins and rapid cooling to 4 °C.

2.4.5 Primer design and preparation

PCR primers for NGF, BDNF, NT-3, trkA, trkB (catalytic isoform), trkC (catalytic isoform), $p75^{NTR}$, nestin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which is used as a housekeeping gene, were designed from published sequences (**Table 2.2**) and synthesised by MWG Biotech AG (Germany) and Sigma (St. Louis, USA). Primers need to be 18-30 nucleotides long; both primers should have similar G + C content in order to have similar annealing temperature, and they were carefully checked by Blast (National Centre for Biotechnology Information, website: http://www.ncbi.nlm.gov/Blast/) to confirm the specificity of these primers and by a software "Generunner (version 3.05, freely downloaded from http://www.generunner.com) to minimise the formation of secondary structures or primary duplexes. The dried primer was prepared to a concentration of 100 pmol/μl (100 mM) by adding appropriate amount of distilled water shown on the product sheet from the suppliers. A 5 mM primer solution was then made by mixing 10 μl the 100mM primer solution into 190 μl distilled water, and aliquots were made and kept at –20 °C.

2.4.6 Polymerase chain reaction (PCR)

PCR is a method of DNA synthesis resulting in the amplification of a fragment of interest from single-stranded DNA template or cDNA libraries. Reactions were carried out in thin-walled PCR tubes in a GeneAmp PCR system 9700 (Applied Biosystems). HotStarTaq

Master Mix Kit (Qiagen) was used and its instruction followed. Preparing a 30 µl reaction mix, 0.6 µl cDNA, 3 µl 5' primer (0.5 µM), 3 µl 3' primer (0.5 µM), 8.4 µl distilled water and 15 µl HotStar Master Mix were added. In all experiments, samples containing no template were included to serve as negative controls. The programme included pre-incubation step at 95°C for 15 min to activate the DNA polymerase, denaturation at 95 °C for 45 seconds, annealing at 55 °C, 58 °C, or 61 °C for 1 min, and extension at 72 °C for 1 min. After 40 cycles, a 10 mins final extension at 72 °C was carried out, followed by cooling to 4 °C.

Table 2.2: Oligonucleotide sequences used in the real-time quantitative PCR reactions

	Forward primer	Reverse primer	GenBank accession number
NGF	ACAGGCAGAACCGTAC ACAG (337-356)	ATCCAGAGTGTCCGAAG AGG (595-576)	M36589
BDNF	CCGCAAACATGTCTAT GAGG (2485-2504)	GATTFFFTAGTTCGGCAT TG (2767-2748)	D10938 (Yun et al., 2002)
NT-3	AGAAGCCAGGCCAGTC AAAA (681-700)	TCCCGAGAGCCCAATCA CAA (1092-1073)	M33968 (Zha et al., 2001)
TrkA	ATGGAGAACCCACAGT ACTTC (1541-1561)	CGTGCAGACTCCAAAGA AGC (1791-1810)	M85214
TrkB (catalytic)	AAAGGCCCAGCTTCCG TCAT (2054-2073)	GGGGGTTTTCAATGACA GGG (2183-2202)	M55291 (Moshnyakov et al., 1996)
TrkC (catalytic)	GGGAAGCAACCATGGT TC (2308-2325)	AAACGCTTGGCCACCAG T (2535-2552)	L03813 (Moshnyakov et al., 1996)
$p75^{NTR}$	TGCAGTGTGCAGATGT GCCT (428-447)	GGGATCTCTTCGCATTCA GC (672-691)	X05137
GAPDH	CCCATCACCATCTTCCA GGAGC (241-262)	CCAGTGAGCTTCCCGTTC AGC (713-693)	NM_017008-1

2.4.7 Preparation of standard samples: agarose gel electrophoresis of DNA

To check for the presence of a PCR product of the expected size and quantity as the result of optimisation of primers, PCR products were separated on agarose gels. A 2 % (w/v) agarose gel was made by dissolving 2.4 g agarose and 5 µl Ethidium bromide, which permits direct visualisation of DNA products with ultraviolet (UV) light, were dissolved into 120ml of 1 × TBE (100mM Tris, 83 mM Boric acid, 1 mM EDTA) in a microwave

(600 w) for 10 minutes. The boiled transparent solution was left to cool before it was poured into a tape-sealed rectangular mould. A comb is positioned onto the sockets at the top of the mould for forming the wells. After the gel was solidified at RT, the comb and the tape seals were removed. The gel was placed into an electrophoresis tank filled with 1 × TBE to cover the surface of the gel and electrodes. 10 µl of each DNA sample was mixed with 2 µl of 6 × loading buffer (0.25 % bromophenol blue, 0.5 % xylene cyanol FF, 3 % glycerol in water) and loaded into the wells. 100 bp size standard markers were also mixed with 1:5 loading buffer and loaded into separate wells for determining the fragment sizes of the PCR products. Agarose gels were run at 110 V for 60- 90 minutes or until the dye front nearly run through the gel. The gel was then placed into a UV box and the relative size of the PCR products was visualised by EagleSight software version 3.2 (Stratagene). The densities of the PCR products from the same primers but with different annealing temperatures were compared to decide the best reaction condition.

2.4.8 Preparation of standard samples: isolation and extraction of DNA fragments from agarose gel

The whole gel was put into a UV box and the desired fragment was excised with a sharp sterile scalpel blade according to the visible fragment. The small block of gel including the fragment of interest was put into a 2 ml autoclaved tube and the instruction of QIAquick Gel Extraction Kit (Qiagen) was followed. The excised piece of gel was weighed for using appropriate amount of reagents during the extraction. Three volume (600µl) Buffer QG was added into the 2 ml tube and incubated at 50 °C for at least 10 mins until the gel was totally dissolved. One volume (200µl) of isopropanol was added, mixed well

and pipetted into a QIAquick Spin column. The mix was centrifuged for 1 min at 14,000 g and the flow-through was discarded. Another 500 µl buffer QG was added and centrifuged for 1 min at 14,000 g to dissolve any residual gel. 750 µl Buffer PE was pipetted into the column and centrifuge for 1 min at 14,000 g for washing the membrane-bound PCR product. After discarding the flow-through, another 1 min centrifugation was carried out to dry the membrane inside the QIAquick column. The column was placed to a new 1.5 ml tube and put in a 37 °C incubator for 5 mins with cap open. For elution of PCR fragments, 30 µl Buffer EB was carefully pipetted directly to the membrane, and centrifuged for 1 min at 14,000 g. The purified PCR product was stored at –20 °C for standard sample preparation.

2.4.9 Preparation of standard samples: agarose gel electrophoresis of PCR templates

Another 2 % (w/v) agarose gel with 14 wells in it was made as described before. 15 µl of each standard DNA sample was mixed with 3 µl of 6 × loading buffer (0.25 % bromophenol blue, 0.5 % xylene cyanol FF, 3 % glycerol in water) and carefully loaded into the wells. 1 µl (0.5 ng) of pBR322 DNA/BsuRI (Fermentas Inc. Hanover, MD, USA) marker was mixed with 1 µl 6 × loading dye and 4 µl distilled water, and loaded into two separate wells for determining the fragment sizes of the PCR products. Agarose gels were run at 110 V for 90 - 120 minutes or until the dye front nearly run through the gel when the one or two bands with the closest size to each PCR product could be clearly separated from other bands. The gel was then placed into a UV box and the relative size the PCR products was visualised by the EagleEye software in the computer. The function of "Integrated density analysis", which was defined as the sum of the pixel values in a selected area, was

used to determine the copy number of the standard sample by comparing to the marker with known amount of DNA. Standards were made by ten-fold serial dilutions of each recovered PCR products in tRNA (10^1-10^6 copies/2μl except GAPDH 10^2-10^7 copies/2μl).

The calculation formula was shown as below:

For example (**Figure 2.2**):

15 μl of PCR products of trkA was loaded with 3 μl loading dye, and electrophoresis was performed with 1 μl (0.5 ng) of pBR322 DNA/BsuRI (company) marker in separate wells.

Integrated density:

TrkA (270bp): 13896

Marker (267bp, 30.5ng in 1 μl): 4352 (left lane), 4330 (right lane)

TrkA: 30.5×(13896÷(4352+4330)/2)=97.63ng (in 15μl) >> 13.02ng trkA PCR products in 2 μl.

Molecular weight of TrkA (270bp)=approximately 270 bp×660 g/bp=178,200g/6.02×10^{23} molecules

>>1 ng=3.38×10^9 molecules

>>2 μl=13.02ng=4.40×10^{10} molecules

Serial dilutions of trkA were prepared for standards:

6 standards were used in the real-time quantitative PCR: 10^6 molecules/2 μl, 10^5 molecules/2 μl, 10^4 molecules/2 μl, 10^3 molecules/2 μl, 10^2 molecules/2 μl, 10^1 molecules/2 μl.

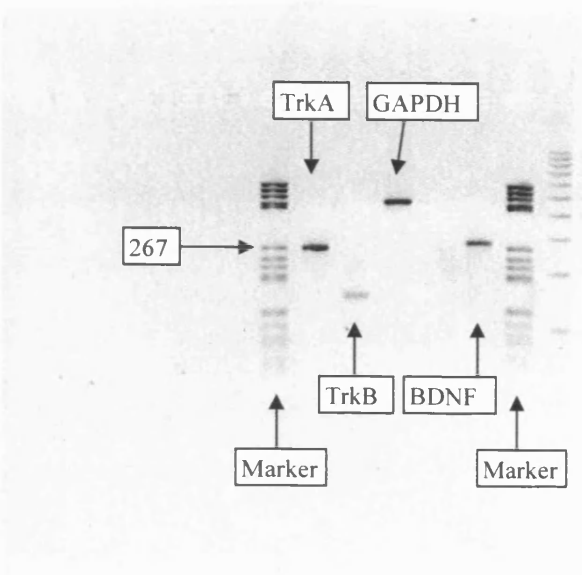


Figure 2.2: Agarose gel electrophoresis of PCR templates.

2.4.10 Gene expression: real-time quantitative PCR

Real-time PCR was performed using a LightCyclerTM rapid thermal cycler system (Roche Diagnostics, Lewes, UK), which is an advanced instrument that conducts rapid thermal cycling of the PCR. It has been proven to be a more sensitive and accurate method for quantification than *in situ* hybridisation, northern blotting, or conventional RT-PCR (Mackay et al., 2002). In brief, SYBR Green I dye, whose fluorescence is greatly enhanced by binding to the minor grooves of the dsDNA, is included in the PCR buffer. After annealing and elongation phase of the PCR, all of the products have become double-stranded, and a maximum amount of dye is bound. The fluorescence is recorded at the end of each elongation phase, and increasing amounts of PCR product can be monitored from cycle to cycle. Extension or termination of runs is also permitted, depending on the conditions.

Reactions were done in a 10μl volume using 5μM primers, dNTPs, FastStart Taq DNA polymerase, DNA double-strand-specific SYBR Green I dye and reaction buffer provided in the LightCycler-FastStart DNA Master SYBR Green I mix. SYBR Green I dye preferentially binds to double-stranded DNA and emits a fluorescent signal proportional to the amount of PCR products. The programme included pre-incubation step at 95°C for 15 min, denaturation at 95°C for 15 seconds, annealing at 55°C (GAPDH), 58°C (TrkA, TrkB, TrkC and BDNF) or 61°C (NGF, NT-3, and $p75^{NTR}$) for 20 seconds, and extension at 72°C for a variable time depending on PCR product size (10-24 seconds). Fluorescent detection to see the real-time amounts of PCR products was carried out at the end of each cycle after a 5-second step 3-5°C below the Tm of product (**Figure 2.3**). Each dsDNA product has its own specific melting temperature (Tm), which is defined as the temperature at which 50% of the DNA becomes single stranded, and 50% remains double stranded. The most important factors determining the Tm are the length and the GC content of PCR product. To confirm amplification specificity, the PCR products from each primer pairs were subjected to a melting curve analysis (**Figure 2.4**) after 40-cycle amplification and subsequent 2% agarose gel electrophoresis. After completion of PCR, the copy number of target genes was calculated by LightCycler software version 3.5.3, which quantifies the sample using extrapolation from the standard curves (**Figure 2.5**). To make the data comparable between animals, the copy numbers of a gene were normalised to a housekeeping gene, GAPDH, before further statistical analysis. The expression of the genes investigated was shown as "copies/10,000 GAPDH copies." The expression in the axotomy + vehicle group was compared with that of the unoperated group in order to detect how these genes were regulated by the injury to the peripheral nerve, and the axotomy +

systemic NT-3 group was compared with axotomy + vehicle group to assess the net effects of systemic NT-3 administration in injured ganglia.

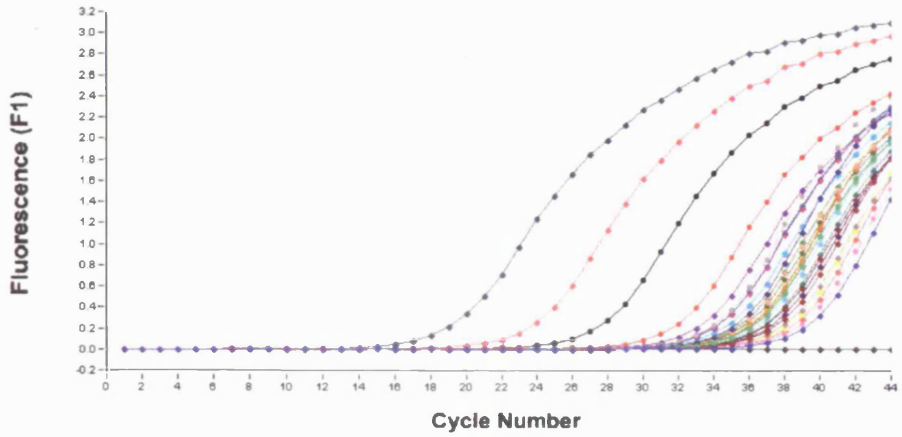


Figure 2.3: Real-time quantitative PCR. The graph shows increase in fluorescence during amplification of trkA using SYBR Green I dye.

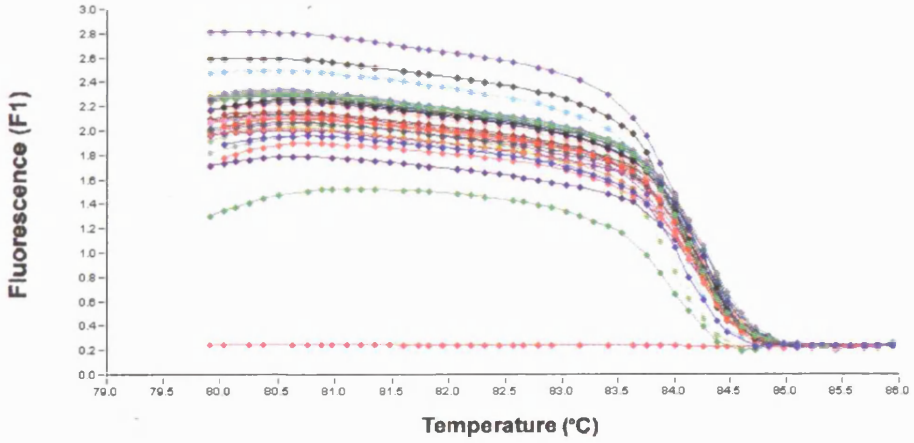


Figure 2.4: Melting curve analysis of trkB PCR fragment.

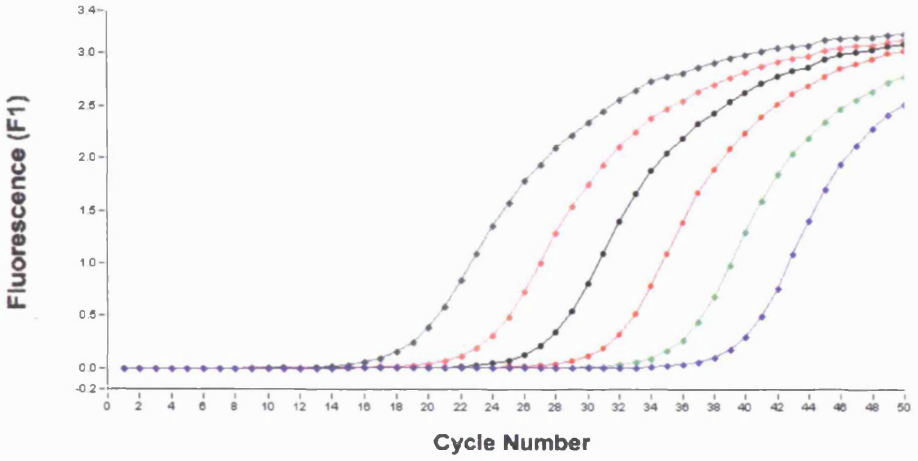


Figure 2.5: Real-time quantitative PCR (standard curves). Fluorescence History Graph displays the development of fluorescence signal intensity in six standard samples of trkA.

2.5 mRNA expression: *in situ* hybridisation

2.5.1 Tissue preparations

14 animals, including 2 animals in each experimental group (1 day, 1 week, 2 weeks) and 2 unoperated controls were perfused transcardially with 4% paraformaldehyde in 0.1M phosphate buffer pH 7.4 at RT for 15 mins under terminal pentobarbitone anaesthesia. The right L4 and L5 dorsal root ganglia were removed and allowed to fix in 10% formalin saline for a further 24 hours before being processed into paraffin. Seven-micrometer thick sections were cut and mounted onto Superfrost slides (BDH) in strips of 4 and dried overnight at 37°C.

2.5.2 Probe preparation

Oligonucleotide probes, purified by HPLC, were synthesised by MWG Biotech AG (Germany) and Sigma (St. Louis, USA). The sequences were complementary to NGF, BDNF, NT-3, trkA, trkB, trkC and $p75^{NTR}$ (**Table 2.3**). The trkB and trkC probes were located in the tyrosine kinase domain to detect the catalytic isoforms of them. The oligonucleotides were labelled at the 3'-end with digoxigenin by using DIG Oligonucleotide 3'-tailing kit (Roche, Lewes, UK), which in average could incorporate 5 DIG molecules in a tail length of 50 to the 3'-end of the probe. In a 20 µl reaction volume, 100 pmol oligonucleotide in 9 µl distilled water, 4 µl of 5 × reaction buffer (1 M potassium cacodylate, 0.125 M Tris-HCl, 1.25 mg/ml bovine serum albumin, pH 6.6), 4 µl of 25 mM $CoCl_2$ solution, 1 µl of 1 mM DIG-dUTP solution, 1 µl of 10 mM dATP solution and 1 µl of terminal transferase (in 0.2 M potassium cacodylate, 1 mM EDTA, 200 mM KCl, 0.2 mg/ml bovine serum albumin) were placed together, well mixed and incubated for 15 minutes in a 37 °C water bath, followed by adding 2 µl 0.2M EDTA (pH 8.0) on ice to stop the reaction.

The DIG-labelled probes were stored in -20°C.

2.5.3 Tissue permeablisation

Paraffin sections were dewaxed in xylene, hydrated in graded alcohol and distilled water. The slides were put level in a plastic container and 150 µl of 20 µg/mL proteinase K (Sigma, St Louis, USA) was applied onto the sections which were incubated in a water bath incubator for 10 minutes at 37°C. The slides were moved to a plastic rack, rinsed in tap water and PBS; before being immersed in 0.1M citrate buffer, pH 6.0, and heated in a 650W microwave oven for 15 minutes. After a brief wash in PBS, the sections were

incubated in100 µl of 1000 units/mL deoxyribonuclease I (Invirogen) for 30 minutes at 37°C.

Table 2.3: Oligonucleotide sequences used in *in situ* hybridisation.

	Probe	GenBank accession number
NGF	CTG CGG GCT CTG CGG AGG GCT GTG TCA AGG GAA TGC TGA AGT TTA GTC CA (447-398)	M36589 (Nosrat et al., 1997)
NGF Control	TGG ACT AAA CTT CAG CAT TCC CTT GAC ACA GCC CTC CGC AGA GCC CGC AG (398-447)	
BDNF	CTC CAG AGT CCC ATG GGT CCG CAC AGC TGG GTA GGC CAA G (2255-2216)	M36589 (Nosrat et al., 1997)
BDNF Control	CTT GGC CTA CCC AGC TGT GCG GAC CCA TGG GAC TCT GGA G (2216-2255)	
NT-3	TTT GTC ATC AAT CCC CCT GCA ACC GTT TTT GAC TGG CCT GGC T (726-684)	M36589 (Nosrat et al., 1997)
NT-3 Control	AGC CAG GCC AGT CAA AAA CGG TTG CAG GGG GAT TGA TGA CAA A (684-726)	
TrkA	AGG GTT GAA CTC AAA AGG GTT GTC CAT AAA GGC AGC CAT GAT G (1231-1189)	M85214 (Meakin et al., 1992)
TrkA control	CAT CAT GGC TGC CTT TAT GGA CAA CCC TTT TGA GTT CAA CCC T (1189-1231)	
TrkB	CCA TTA TTC ATA TGA GTG GGG TTA TCC AGC TGG AGG CAG CCG TGG (1734-1690)	M55291 (Middlemas et al., 1991)
TrkB Control	CCA CGG CTG CCT CCA GCT GGA TAA CCC CAC TCA TAT GAA TAA TGG (1690-1734)	
TrkC	TCC AAA GGC TCC CTC ACC CAG TTC TCT CTT CAA CAC GAT GTC TCT (1701-1657)	L03813 (Merlio et al., 1992)
TrkC control	AGA GAC ATC GTG TTG AAG AGA GAA CTG GGT GAG GGA GCC TTT GGA (1657-1701)	
$p75^{NTR}$	CAC AAG GCC CAC GAC CAC AGC AGC CAA GAT GGA GCA ATA GAC AGG (918-873)	X05137 (Radeke et al., 1987)
$p75^{NTR}$ control	CCT GTC TAT TGC TCC ATC TTG GCT GCT GTG GTC GTG GGC CTT GTG (873-918)	

2.5.4 Hybridisation

Hybridisation mix without probes was preheated to 37 °C. For prehybridisation, sections were covered with 100μL hybridisation mix, containing 2 × SSC, 5% dextran sulfate, 10% formamide, 200μg/ml SSDA and 0.1 mg/ml poly [A] (Roche), for 30 minutes at 37°C. Poly [A] was used to block the non-specific hybridisation of the DIG-dUTP/dATP tail to related homologous sequences. Then, the prehybridisation mix was poured off and 100 μl hybridisation mix containing 0.5μg/mL sense (used as a negative control) or antisense probe and prehybridisation solution was applied gently to the slides. The slides were carefully covered with Parafilm membrane which was trimmed to fit the size of the section, avoided of any air bubbles and put into a tape-sealed plastic container incubated in a water bath incubator for 16 h at 37°C.

Post-hybridisation washes were undertaken as follows: Rinse in 1 × SSC once for 2 minutes at RT, 1 × SSC for 15 minutes at 55 °C, 0.5 × SSC for 15 minutes at 55 °C, and once in 0.5 × SSC for 10 minutes at RT. Non-specific binding sites were blocked in 10% milk for 15 minutes at RT, followed by an 1 : 200 alkaline phosphatase-coupled sheep anti-DIG IgG (Roche), which binds to the DIG label of the oligonucleotide probe, in 2% milk for 30 minutes at room temperature. The anti-DIG-AP antibody was then washed away by 3 washes in TBST buffer (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 5 minutes at RT.

There were a number of controls added to my experiment to verify that the probes used were in fact binding selectively to the target mRNA sequences and not to other components of the cell or other closely related mRNA sequences. The tissue was digested with RNases prior to hybridisation with the oligonucleotide probe. The absence of binding after RNase treatment indicates that binding was indeed to RNA within the tissue. The

sense control probe gives a measure of non-specific probe binding only due to the chemical properties of the probe.

2.5.5 Detection

The colour reaction was developed by NBT/BCIP, after 5 minutes incubation in 1 × detection buffer (0.1 M Tris-HCl, 0.1M NaCl, pH 9.5) at RT. The alkaline phosphatase substrate consists of 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitro blue tetrazolium chloride (NBT). In a 20 ml tube protected from light with foil, one tablet of NBT/BCIP (Roche) was dissolved in 10 ml distilled water with 100 µl levamisole (20mg/ml, Sigma). Approximately 300 µl of AP substrate was the placed per slide. After appropriate time of reaction (3-16 hours), sections were washed with water, dehydrated in graded alcohol and mounted in aqueous mounting medium and examined under a light microscope. Under microscopy, the type of cells (neurons, Schwann cells, or satellite cells...) expressing a certain mRNA was identified and recorded to assist the data analysis from real-time PCR study.

2.6 mRNA expression: studied by microarray

2.6.1 Tissue preparation

24 experimental rats with NT-3 or vehicle subcutaneous pump were sacrificed at 2 weeks after operation by deep anaesthesia with pentobarbitone (60mg/kg, i.p.); 12 additional unoperated animals provided control DRGs. The right L4 and L5 dorsal root

ganglia were removed rapidly and kept in RNAlater solution (Qiagen) on ice. These ganglia were cut thoroughly and total RNA was isolated using QIAshredder spin column, RNeasy Mini Kit and DNase Set (Qiagen) as described in 2.3.1. RNA was assessed quantitatively by spectrophotometry and precipitated, using sodium acetate, pH 4.0, and isopropanol at -20°C overnight, and then resuspended in appropriate amount of RNase-free water. Triplicate measurements were carried out on different DRG tissue. Each RNA sample used for hybridisation of each array was extracted from rat right L4 and L5 DRGs (8 ganglia were pooled from 4 animals).

RNA samples were analysed by denatured gel electrophoresis and RNA quality was assessed by capillary electrophoresis (Bioanalyzer 2100 Agilent, Palo Alto, CA, USA) to ensure the 28S:18S rRNA ratio was > 1.0 for each sample.

2.6.2 Quantification of total RNA

RNA 6000 Nano LabChip Kit (Agilent Technologies) and Agilent 2100 bioanalyzer were used, following the maker's instructions. 400 µl RNA 6000 Nano gel matrix was placed into a spin column and centrifuged at 4000 rpm (1500 × g) in an Eppendorf microcentrifuge for 10 minutes. 2 µl RNA 6000 Nano dye concentrate was added to 130 µl of filtered gel matrix, vortexed (model MS2-S8) and then centrifuged at 14,000 rpm (13,000 × g) for 10 minutes at 4 °C. 9 µl of the gel-dye mixture was properly pipetted to each of the three wells marked with "G", and the chip was primed using Chip Priming Station to plunge 1 ml air for 30 seconds over the wells applied with gel-dye mixture. 5 µl RNA 6000 Nano Marker was pipetted into all the wells except the three filled with gel-dye mix. RNA 6000 ladder (Ambion Inc., cat. No. 7152) and all RNA samples were incubated

at 70 °C for 2 minutes to prevent secondary structures. 1 μl of the each RNA sample and the ladder was pipetted into sample wells and the ladder well. Then the chip was vortexed for 1 minute at the IKA Vortex Mixer (model MS2-S8/MS2-S9). The assay started to be analysed after inserting the chip into the Agilent 2100 Bioanalyzer. The results of samples were shown by electropherogram and the quality and concentration of RNA was determined.

2.6.3 First strand cDNA synthesis

In a thin-walled PCR tube, 15 μl total RNA was added with 2 μL oligo[$(dT)_{24}$ T7 promotor]$_{65}$ primer (100pmol/μl)(5'-GGC CAG TGA ATT GTA ATA CGA CTC ACT ATA GGG AGG CGG-$(T)_{24}$-3') and 4 μl distilled water, denatured at 70°C for 10 min and quick-chilled on ice for 2 minutes. After adding 8 μL 5× first strand RT-buffer (50mM Tris, 250mM KCl; pH 8.3), 4 μl 0.1M DTT (0.1M), 4 μl dNTP (10 mM each), and 1 μl RNase inhibitor (25 U/μL) to the 21 μl RNA/primer mixture, first strand cDNA synthesis was performed at 42°C for one hour using 2 μl AMV Rerverse Transcriptase (25 U/μl). The reaction was terminated by placing on ice.

2.6.4 Second strand cDNA synthesis

Adding 72 μl DEPC-treated water, 30 μl 5× second strand buffer, 1.5 μl 10mM dNTP mix (10 mM each), 6.5 μl second strand enzyme blend (*E. coli* DNA Polymerase I, *E. coli* DNA ligase and RNase H) to the 40μl first strand reaction to make a final volume of 150 μL. Second strand cDNA synthesis was done at 16°C for 2 hours and followed by 20

μl T4 DNA Polymerase (1 U/μL) at 16°C for another 5 minutes to fill in the ends of the double-stranded cDNA. The reaction was stopped by adding 17 μl EDTA (0.2 M, pH 8.0).

2.6.5 Cleanup of double-stranded cDNA

GeneChip Sample Cleanup Module (Qiagen) was used. 600 μl cDNA Wash Buffer (prepared by adding 24 ml 100% ethanol to 6 ml concentrate of cDNA Wash Buffer) was added to the final preparation of double-stranded cDNA synthesis and vortexed briefly. The pH value was adjusted by adding 3M sodium acetate if the colour of the mixture was not yellow. 500 μl of the mixture was loaded to the cDNA Cleanup Spin Column and centrifuged at 10,000 rpm (8,000 × g) for 1 minute at RT. The flow-through was discarded and the remaining 150 μl of mixture was loaded and spun. The Cleanup spin column was transferred to a 2 ml autoclaved tube, and another 750 μl cDNA Wash Buffer was added and centrifuged at 10,000 rpm (8,000 × g) for 1 minute at RT. 5 minutes of centrifugation was carried out with the cap of the spin column opened to ensure complete drying of the membrane. The spin column was transferred into a 1.5 ml collection tube, and 14 μl of cDNA Elution Buffer was applied directly onto the membrane. After incubation at RT for 1 minute, cDNA was eluted by centrifuging the column at 12,500 rpm (10,000 × g).

2.6.6 Synthesis of biotin-labelled cRNA

Double-stranded cDNA was transcribed *in vitro* into the copy RNA (cRNA) labelled with biotin-UTP and biotin-CTP using Enzo BioArray HighYield RNA Transcript Labeling Kit (Affymetrix). An IVT cRNA labelling mix was prepared (12 μl double-stranded cDNA, 10 μl distilled water, 4 μl 10 × HY Reaction Buffer, 4 μl 10× Biotin-

labelled Ribonucleotides, 4μl 10 × DTT, 4 μl 10 × RNase Inhibitor Mix and 2 μl 20 × T7 RNA polymerase) at RT, and incubated for 5 hours in a 37 °C water bath with mixing gently every 45 minutes. The biotin-labelled cRNA was proceeded to cleanup procedure.

2.6.7 Cleanup of Biotin-labelled cRNA

60 μl of RNase-free water was added to the 40 μl in vitro transcription reaction and mixed briefly. 350 μl IVT cRNA Binding Buffer was pipetted to the tube and well mixed, followed by adding 250 μl 100% ethanol and pipetting to mix. The total 700 μl of the mix was then applied to the IVT cRNA Cleanup Spin Column and centrifuged at 10,000 rpm (8,000 × g) for 15 seconds at RT. The column was transferred to a new 2 ml tube and washed by 500 μl IVT cRNA Wash Buffer at 10,000 rpm (8,000 × g) for 15 seconds at RT and 500 μl 80% (v/v) ethanol at 10,000 rpm (8,000 × g) for 15 seconds. The spin column was centrifuged for another 5 minutes at 13,200 rpm (10,000 × g) to dry the membrane. The spin column was transferred to a new 1.5 ml collection tube and 21 μl RNase-free water was applied directly onto the membrane and biotin-labelled cRNA was eluted after centrifuging for 1 minute at 13200 rpm.

2.6.8 Quantification of Biotin-labelled cRNA

RNA 6000 ladder (Ambion Inc., cat. No. 7152) and all biotin-labelled cRNA samples were incubated at 70°C for 2 minutes to prevent secondary structures. 1 μl of a 1:10 dilution of each cRNA sample and the ladder was pipetted into sample and ladder wells of RNA 6000 Nano LabChip Kit. The assay was analysed using the Agilent 2100 Bioanalyzer as described before. The concentration of RNA was determined.

2.6.9 Fragmentation of Biotin-labelled cRNA

2 µl of 5 × Fragmentation Buffer was added in the 8 µl biotin-labelled cRNA and incubated at 94 °C for 35 minutes for breaking cRNA to 35-200 base fragments by metal-induced hydrolysis. The reaction was stopped by putting on ice.

2.6.10 Target hybridisation

Hybridisation buffer (12 × MES: 100 mM MES [70.4 g MES-free acid monohydrate, 193.3g MES sodium salt in 1000ml water], pH 6.5-6.7, 1 M NaCl, 0.01% Tween 20, 20 mM EDTA, 0.5 mg/ml acetylated bovine serum albumin (BSA), 0.1 mg/ml herring sperm DNA, 50pM control oligonucleotide B2, and eukaryotic hybridisation controls [1.5 pM *bioB*, 5 pM *bioC*, 25 pM *bioD* and 100pM *cre*]) was added to 34 µl fragmented cRNA of each sample, resulting the final volume of 100 µl. Pre-hybridisation of the arrays was performed with 1 × Hybridisation Buffer at 45 °C for 10 minutes with rotation in Hybridisation Oven 640 (Affymetrix), and the hybridisation cocktail was heated for denaturation at 99 °C for 5 minutes, followed by equilibration at 45 °C for 5 minutes. Samples were then hybridised to Rat Neurobiology U-34 microarrays (Affymetrix) at 45°C for 16 hours using Hybridisation Oven 640.

2.6.11 Washing and staining

Microarrays were washed with non-stringent Wash Buffer A (6 × SSPE [20 × SSPE: 3M NaCl, 0.2M NaH_2PO_4, 0.02M EDTA], 0.01% Tween 20) at 25 °C for 10 cycles of 2 mixes/cycle and then stringent Wash Buffer B (100mM MES, 0.1M [Na^+], 0.01% Tween

20) at 50 °C for 4 cycles of 15 mixes/cycle using the Fluidics Station 400. The arrays were incubated with SAPE solution (100 mM MES, 1M [Na^+], 0.05% Tween 20, 50 mg/ml acetylated BSA, 1mg/ml streptavidin phycoerythrin (SAPE) and distilled water) at 25 °C for 10 minutes, followed by 10 cycles of 4 mixes/cycle wash with Wash Buffer A. The probe arrays were then sequentially incubated with Antibody Solution Mix (100 mM MES, 1M [Na^+], 0.05% Tween 20, 2 mg/ml acetylated BSA, 0.1 mg/ml normal goat IgG, 3 μg/ml biotinylated antibody and distilled water) at 25 °C for 10 minutes, and SAPE solution at 25 °C for 10 minutes. Final wash was performed with Wash Buffer A at 30 °C for 15 cycles of 4 mixes/cycle.

2.6.12 Scanning

The Agilent HP G2500A GeneArray Scanner, controlled by Affymetrix Microarray Suite, was used to scan the probe arrays (**Figure 2.6**). Multiple distinct oligonucleotide probes on each chip represent every gene, and the intensity and colour of each spot encode information on a specific gene from the tested sample. Relatively low background and high and uniform signals in this image provide a basis for comparison of the microarrays. The intensity of signal for genes was adjusted to 100 so that the mean intensities for each array were equal.

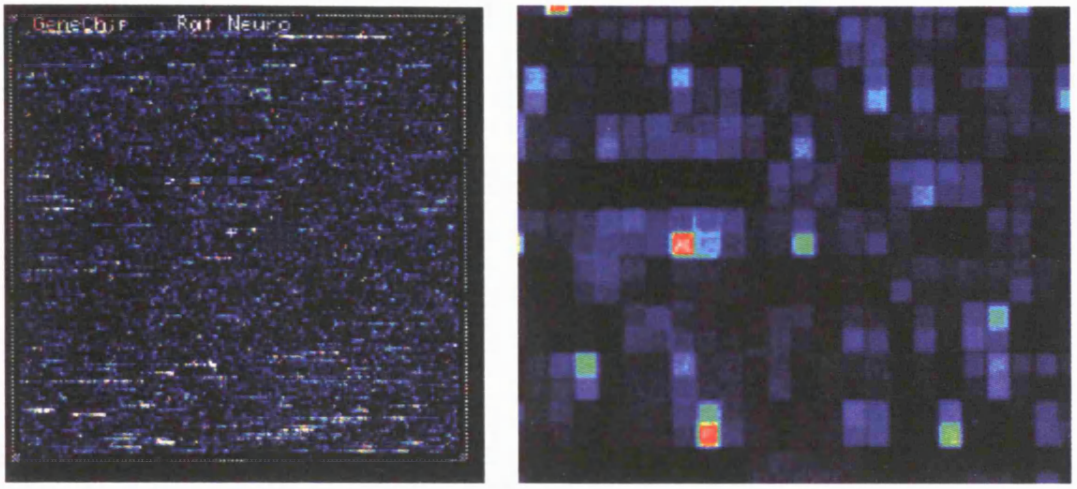


Figure 2.6: Scanned images of a neurobiology U-34 microarray.

2.6.13 Data analysis

The data were collected and globally scaled to a target intensity of 100 using Affymetrix Microarray Suite 5.0. Pairwise comparisons were generated using Microarray Suite and analysed using Affymetrix MicroDB and DataMiningTool 3.0. The other data analysis was performed using Genespring 5.1 (Silicon Genetics).

2.6.14 Confirmation by real-time quantitative PCR

PCR primers for neurofilament-light chain (NF-L), neuropeptide Y (NP-Y), 5HT3 receptor (5HT-3R), $p75^{NTR}$, heat shock protein (HSP) 70, peripheral-type benzodiazepine receptor, glutamate receptor subnit 5-2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which is used as a housekeeping gene, were designed from published sequences (**Table 2.4**). Conventional PCR reactions were performed in a 30µl solution using HotStarTaq Master Mix Kit (Qiagen) to optimise the condition. In all experiments, samples containing no template were included to serve as negative controls.

Table 2.4: Oligonucleotide sequences used in the real-time quantitative PCR reactions for microarray confirmation.

	Forward primer	Reverse primer	GenBank accession number
NF-L	ATGCTCAGATCTC CG TGGAGATG (790-812)	GCTTCGCAGCTCA TTCTCCAGTT (1154-1132)	AF031880 (Chidlow and Osborne, 2003)
NP-Y	TCCAGCCCTGAGA CACTGATT (273-293)	CACCACATGGAA GGGTCTTCA (364-344)	M15880 (Song et al., 2001)
5HT-3R	ACG CTC CTT CTG GGA TAC TCA GT (850-872)	TGCACACTACAAA GTAGACACCAATG (952-927)	D49395 (Wang et al., 2002a)
$P75^{NTR}$	TGCAGTGTGCAGAT GTGCCT (428-447)	GGGATCTCTTCGCA TTCAGC (672-691)	X05137
HSP70	CAAGAATGCGCTC GAGTCCTA (1791-1811)	GGAGATGACCTCC TGGCACTT (1914-1894)	L16764 (Song et al., 2001)
Peripheral-type benzodiazepine receptor	TGGTATGCTAGCT TGCAGAAACC (131-153)	CGAATACAGTGTG CCCCAGAT (208-188)	J05122 (Wang et al., 2002a)
Glutamate Receptor subunit5-2	CGGCATGAATTAA GAAGCTTGAA (163-185)	CCTCCGATCCTGA GCACTTG (309-290)	M83561 (Wang et al., 2002a)
GAPDH	CCCATCACCATCTT CCAGGAGC (241-262)	CCAGTGAGCTTCCC GTTCAGC (713-693)	NM_017008-1

2.7 Statistics

The individual values for neuron number, neuronal loss (right to left ratios) and apoptosis for the animals in the two treatment groups, and the operated and unoperated control groups, as well as the relative gene expression data between the different treatment groups and the unoperated control group were analysed using one-way ANOVA to check whether the groups of rats came from a population with the same mean followed by Tukey's *post hoc* tests at 95% confidence limits to identify the existence of significant difference between any two groups. All data are given as mean ± SEM. The difference between the absolute neuronal numbers of right and left L4&L5 DRGs in each group was analysed using paired-t test. These were performed using SPSS 11.5 for Windows on an IBM-PC.

CHAPTER 3: RESULTS

3.1 Effect of NT-3 on axotomy-induced neuron loss in adult rat dorsal root ganglia

Animals receiving NT-3 appeared to tolerate it well and increased in weight to an identical degree over 4 weeks when compared to those receiving vehicle. Using ANOVA analysis, there was no difference in weight of rats ($p>0.05$, **Table 3.1**). In addition, there was no noticeable difference in behaviour such as keeping the affected limb raised or in the incidence of autotomy between NT-3 and vehicle-treated animals. Macroscopic examination of heart, lungs, kidney, liver and gastrointestinal tract did not reveal any abnormalities in any group.

3.1.1 Morphology

In the ipsilateral ganglia of all operated groups, the number of non-neuronal cells increased and numerous chromatolytic neurons with eccentric nuclei, marginal Nissl staining and a slightly shrunken appearance were observed (**Figure 3.1**), when compared to unoperated rat ganglia. At 4 weeks after sciatic nerve transection the histological appearance of the ipsilateral ganglia from both NT-3-treated groups was not substantially different from that of the vehicle-treated or transection/no treatment groups, with many neurons having eccentric nuclei, marginal Nissl staining and a slightly shrunken appearance typical of chromatolysis produced by axotomy.

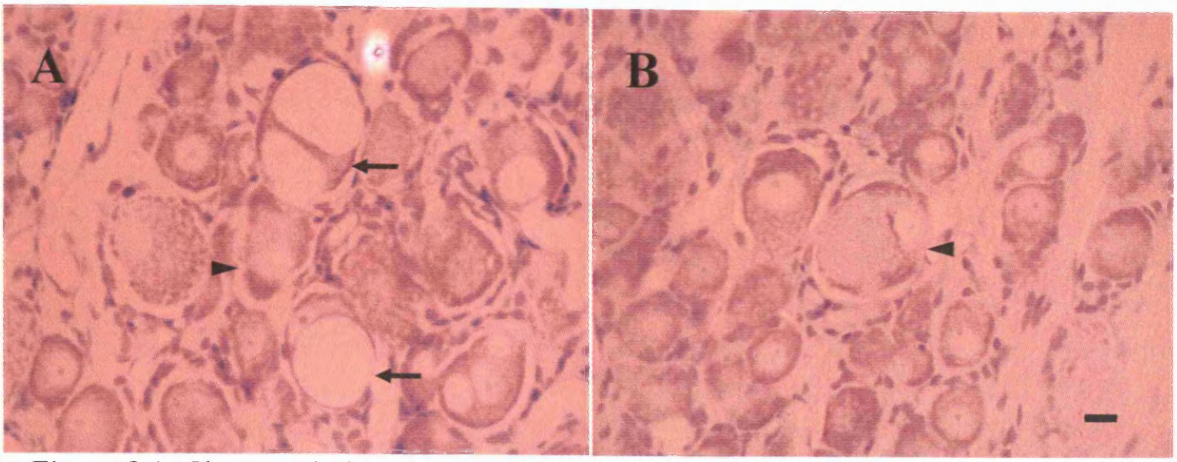


Figure 3.1: Chromatolytic neurons (arrowheads) 4 weeks after axotomy with vehicle administration, stained with cresyl fast violet. Some vacuolated neurons are visible (arrows). Scale bar = 10μm

3.1.2 Neuronal number in axotomised DRG

The results of neuronal counting, using the stereological physical disector technique on 3 μm sections of the L4 and L5 DRGs from all 7 experimental groups of animals are summarised in **Table 3.1**. The thickness of each examined section was checked using a microcator as described in 2.2.3.

Analysed by one-way ANOVA, there was no significant difference in the mean total number of neurons in left L4 and L5 dorsal root ganglia (pooled) in any of the experimental groups (ANOVA: $p>0.05$). The ratio of neuron numbers between right (L4 & L5) and left (L4 & L5) in unoperated rats shows a consistently and significantly (paired t-test: $p=0.016$) greater number (about 7%) of neurons on the right side than on the left. An asymmetry in rat DRG neuron number has been reported (Ygge et al., 1981; Arvidsson et al., 1986; Schmalbruch, 1987b).

The ratio of numbers of neurons in the ipsilateral L4 and L5 DRGs to those in the contralateral DRGs (**Figure 3.2**) show that there was no significant loss of neurons in the ipsilateral ganglia 2 weeks after mid-thigh sciatic nerve transection and ligation, in either

NT-3-treated or vehicle-treated groups when compared to unoperated controls (ANOVA: $p=0.069$). In the 4 week axotomy without treatment and 4 week axotomy with vehicle administration groups, there was a statistically significant neuronal loss (Tukey's *post hoc* test: $p<0.05$) of around 16% after peripheral axotomy.

Table 3.1: Mean neuron numbers and mean ratios of neuron numbers between ipsi- (right) and contralateral (left) L4 and L5 DRGs in each treatment group. The ratios of neuron numbers between the different treatment groups and the unoperated control group, and weight of rats (before tissue preparation) were analyzed using one-way ANOVA followed by Tukey's *post hoc* tests (Appendix 1).

Group (Weight of rats: mean ± SD)	**Mean number of neurons in left L4 & L5 (± SEM)**	**Mean number of neurons in right L4 & L5 (± SEM)**	**Mean R:L ratio in neuronal number (± SEM)**	**P value (Tukey's *post hoc*, compared to unoperated)**
Unoperated rats, n=7 (293.6 ± 24.45)	25998 ± 904	27800 ± 1036	1.070 ± 0.019	
2 weeks sciatic transection with vehicle pump, n=4(313.75 ± 29.38)	26826 ± 853	27128 ± 1151	1.015 ± 0.055	0.579
2 weeks sciatic transection with NT-3 (0.625mg/2 weeks) pump, n=5 (308.8 ± 24.89)	29335 ± 1556	27290 ± 653	0.938 ± 0.044	0.057
4 weeks axotomy, n=5 (327.6 ± 16.44)	28976 ± 1431	25995 ± 912	0.898 ± 0.030	0.013*
4 weeks sciatic transection with vehicle pump, n=5 (324.2 ± 24.65)	25922 ± 617	23608 ± 1017	0.912 ± 0.043	0.025*
4 weeks sciatic transection with NT-3 (1.25mg/4 weeks) pump, n=5 (334.0 ± 26.22)	26387 ± 731	26757 ± 504	1.019 ± 0.045	0.817

4 weeks sciatic transection with NT-3 (5mg/4 weeks) pump, n=4 (333.4 ± 44.29)	27599 ± 1129	28693 ± 1317	1.042 ± 0.038	0.979

* indicates $p<0.05$, compared with unoperated group.

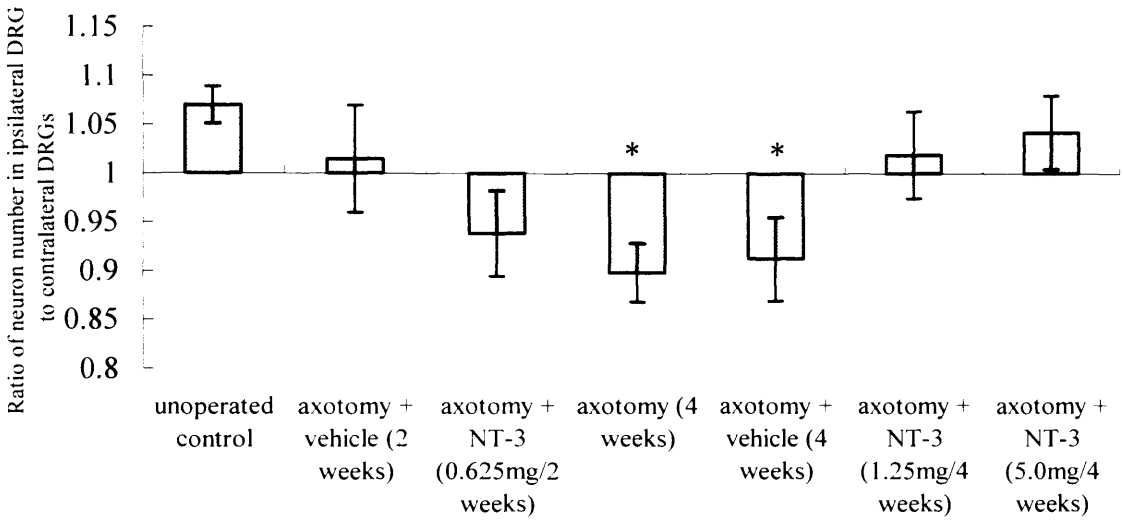


Figure 3.2: Mean ratios (± SEM) of neuron number between right and left L4 and L5 DRGs for each treatment group. * indicates $p < 0.05$ compared with vehicle-treated group

3.1.3 Effect of NT-3 on neuron number in axotomised DRG at two doses

In contrast to the significant neuronal loss occurring in the 4-week axotomy without treatment group and the 4-week axotomy with vehicle administration group, NT-3-treated groups at the dose of either 1.25mg/4 weeks or 5mg/4 weeks suffered no significant neuronal loss of ipsilateral neurons, relative to the contralateral. There was no significant difference between the rats treated with 1.25mg and 5mg NT-3 for 4 weeks after axotomy ($p=0.996$). These data show that systemic NT-3 administration preserves the R : L ratio in neuron number in L4 and L5 DRGs for up to 4 weeks following injury.

4 weeks sciatic transection with NT-3 (5mg/4 weeks) pump, n=4 (333.4 ± 44.29)	27599 ± 1129	28693 ± 1317	1.042 ± 0.038	0.979

* indicates $p<0.05$, compared with unoperated group.

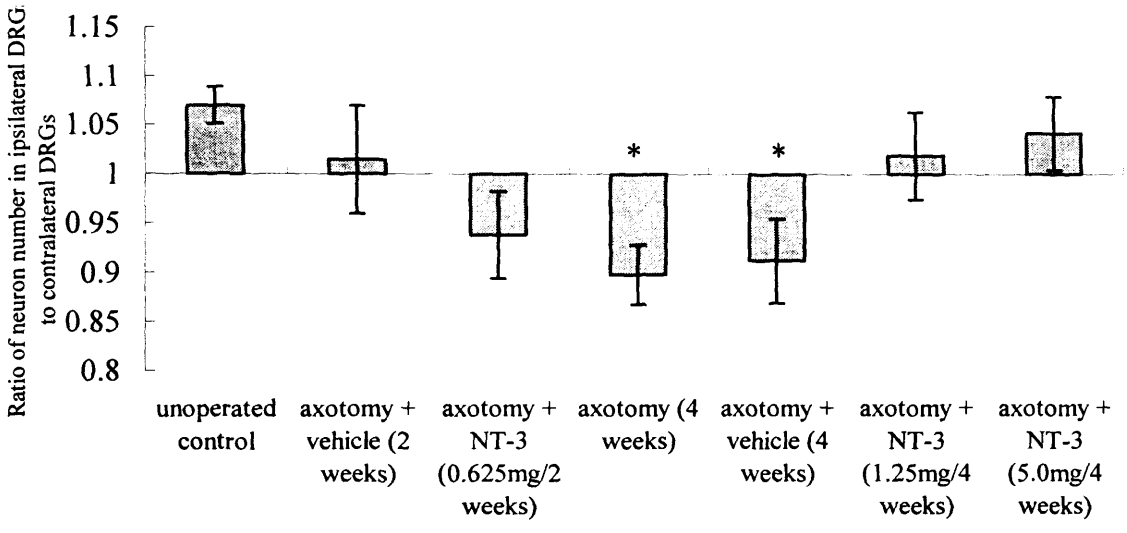


Figure 3.2: Mean ratios (± SEM) of neuron number between right and left L4 and L5 DRGs for each treatment group. * indicates $p < 0.05$ compared with vehicle-treated group

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3.2 Effect of NT-3 on axotomy-induced neuron apoptosis in adult rat dorsal root ganglia

Neuronal apoptosis in DRG has been previously reported and described following sciatic nerve transection in adult rat and mouse (Ekstrom, 1995; Groves et al., 1997; McKay Hart et al., 2002), but whether systemic NT-3 administration can affect the morphological features of neurons or the incidence of neuronal apoptosis in axotomised DRG has never been studied. This section reports the number of profiles of apoptotic neurons assessed by morphological and immunohistochemical criteria 2 and 4 weeks after mid-thigh sciatic nerve transection with no treatment, systemic vehicle administration or systemic NT-3 administration at two different doses.

3.2.1 Morphology

Profiles of apoptotic neurons in DRG were identified by morphological criteria. The key criterion was the condensation of DNA into several spherical condensed bodies. Secondary criteria were indistinct and irregular nuclear membrane, and darkly staining cytoplasm with condensed Nissl substance and shrunken appearance. There were some variations in morphology, such as the number, size and distribution of condensed nuclear chromatin bodies (**Figure 3.3**). Structures 15-30 μm in diameter that were lacking any visible cellular contents except for the remains of some condensed DNA, and which were surrounded by satellite cells, corresponded to the "ghost cells" (**Figure 3.4**) reported from cell culture studies of sympathetic neurons undergoing apoptosis (Edwards and Tolkovsky, 1994). Ghost cells were seen particularly at the 2 week time point. Due to the difficulty to identify the origin of these cells, they were not counted as either normal or apoptotic

neurons in this study. Neurons undergoing apoptosis in the NT-3-treated animals did not differ in morphology from those in the vehicle-treated rats.

Neither apoptotic or degenerating neurons, nor any evidence of neuronophagia or neuronal activated caspase-3 immunoreactivity were seen in any of the serial sections of unoperated or contralateral DRGs from any group. These data indicate that there is no detectable neuronal apoptosis occurring in normal DRGs or contralateral DRGs in operated animals, which makes it reasonable to estimate neuronal loss by the ratio of neuron number between right (L4 & L5) and left (L4 & L5) DRGs.

3.2.2 Incidence of neuron apoptosis

Each section was estimated at a magnification of ×400 under light microscope for profiles of apoptotic neurons. The total number of profiles of apoptotic neurons in half of the sections of each ganglion were counted and this figure was multiplied by 2 to obtain an estimate of the total per ganglion.

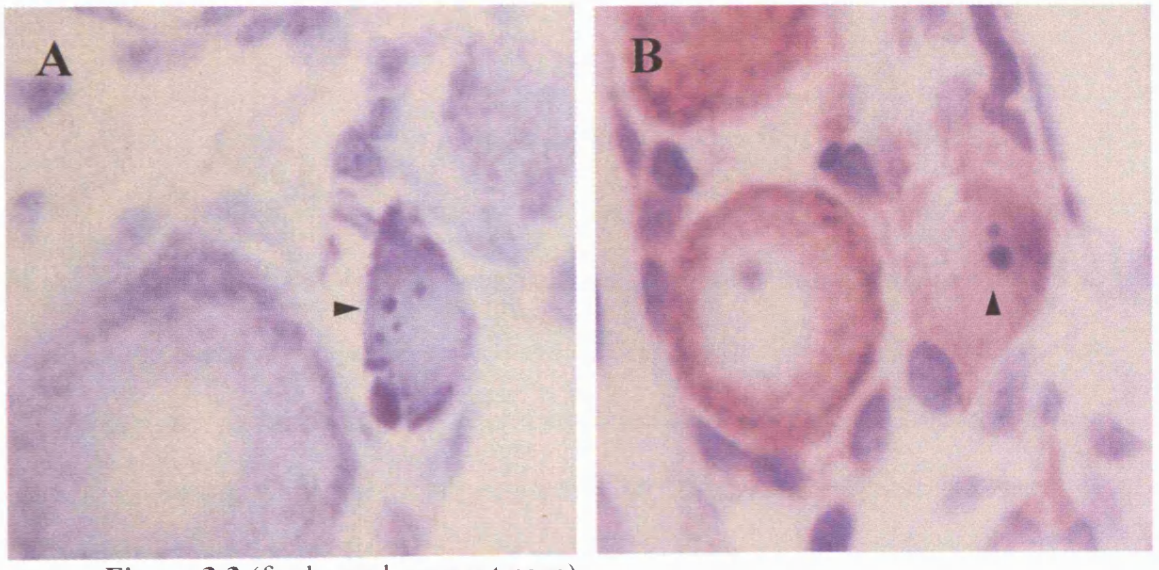


Figure 3.3 (for legend see next page)

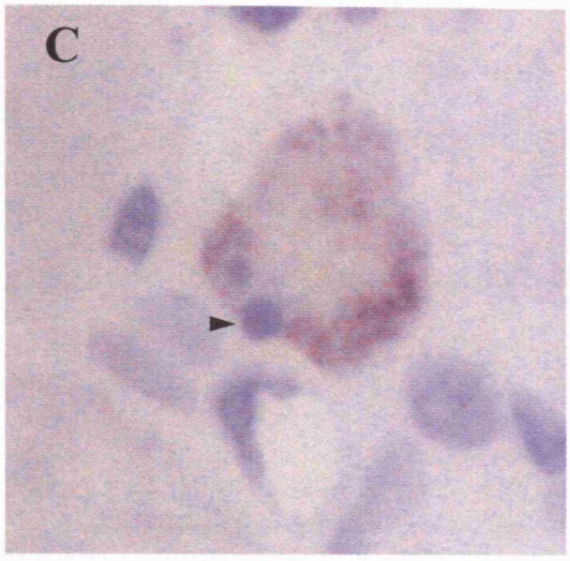


Figure 3.3: Apoptotic DRG neurons 2 weeks after axotomy with vehicle administration, stained with cresyl fast violet (A, B) and anti-activated caspase-3 antibody (C). These neurons have a shrunken and condensed shape and the characteristic condensed chromatin (arrowhead). The grey-purple staining of activated caspase-3 was detected in both nucleus and cytoplasm.

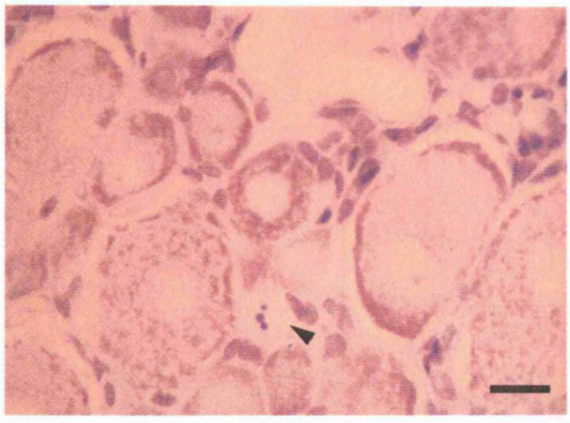


Figure 3.4: A 'ghost cell' (arrowhead) 4 weeks after axotomy with vehicle administration, stained with cresyl fast violet. Scale bar = 10μm

The number of profiles of apoptotic neurons ranges from 10.6-19.4 in pooled L4 & L5 DRGs (**Table 3.2**). When the data were analysed by performing ANOVA, the number of profiles of neurons showing the morphological characteristics of apoptosis did not differ significantly between any of the experimental groups ($p=0.59$, **Figure 3.5**). All neurons identified as apoptotic by their morphology showed activated caspase-3 immunoreactivity in sections immunostained for it (**Figure 3.3C**). My results showed that there is no significant difference between the number of profiles of apoptotic neurons at 2 weeks in

NT-3-treated and vehicle-treated groups, and that NT-3 treatment did not significantly delay the onset of neuron apoptosis, which normally starts during the first week after axotomy (Groves et al., 1997; McKay Hart et al., 2002). NT-3 administration for 4 weeks at both doses also had no significant effect on profiles of apoptotic neurons, when compared to the vehicle-treated group or no treatment group.

Table 3.2: The number of profiles of apoptotic neurons in ipsilateral L4 and L5 DRGs in each treatment group (Mean ± SEM). The estimated number between the different treatment groups and the unoperated control group were analyzed using one-way ANOVA followed by Tukey's *post hoc* tests (Appendix 1). (ANOVA: p=0.59)

Group	**Number of** profiles of apoptotic neurons **in ipsilateral L4 & L5 (Mean ± SEM)**
Unoperated rats (n=7)	0
2 weeks sciatic transection with vehicle pump (n=4)	15.5 ± 3.0
2 weeks sciatic transection with NT-3 (1.25mg/4 weeks) pump (n=5)	19.4 ± 4.8
4 weeks sciatic transection (n=5)	16.0 ± 2.7
4 weeks sciatic transection with vehicle pump (n=5)	14.4 ± 3.4
4 weeks sciatic transection with NT-3 (1.25mg/4 weeks) pump (n=5)	10.6 ± 3.2
4 weeks sciatic transection with NT-3 (5mg/4 weeks) pump (n=4)	13.0 ± 3.1

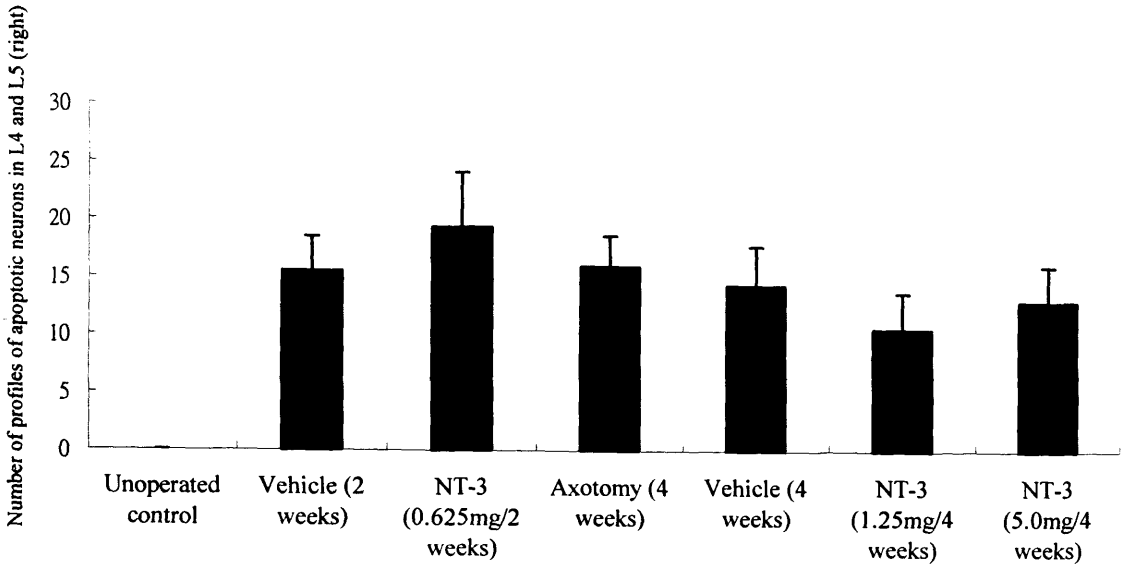


Figure 3.5: The number of profiles of apoptotic neurons in ipsilateral L4 and L5 DRGs in each treatment group (Mean ± SEM).

3.3 Cell size profile in axotomised dorsal root ganglion with and without NT-3 administration

3.3.1 Cell size profile in normal dorsal root ganglion

The cross-sectional area of sensory neurons in bilateral L4 and L5 DRGs was measured in three rats of each group studied; the neuronal diameter was calculated and the size frequency distribution histograms are shown in **Figure 3.6**. Adult rat sensory neurons vary enormously in size, with diameter ranges from 12μm to 65μm. Most of the cells appear to be round or oval. In unoperated animals, the size profile has a bimodal distribution, one peak at 26-30 μm and one at 42-46 μm, showing that there are at least two main subpopulations: one of smaller and one of larger neurons with some intermediate-size

neurons (**Figure 3.6A**). The smallest neurons are 12-14 μm in diameter, and the biggest ones are more than 60 μm.

3.3.2 Cell size profile in axotomised DRG

Two weeks after right sciatic nerve transection and vehicle administration, the size-frequency histogram showed an obvious shift towards the left, indicative of a general decrease in cell size compared to the contralateral ganglia (**Figure 3.6B**). The bimodal distribution was grossly preserved, but the two peaks moved to 20-22 μm and 34-38 μm, respectively (**Figure 3.6B**).

Four weeks after axotomy (**Figure 3.6C**), the distribution of cell size appeared to be similar to that of two week axotomy group.

3.3.3 Effect of NT-3 on neuronal size distribution in axotomised DRG

Systemic NT-3 administration for 2 weeks and 4 weeks at the dose of 1.25mg/4 weeks or 4 weeks at the dose of 5mg/4 weeks appeared to have no effect on the leftward shift of the size frequency distribution of neurons after peripheral axotomy (**Figure 3.6D, E & F**).

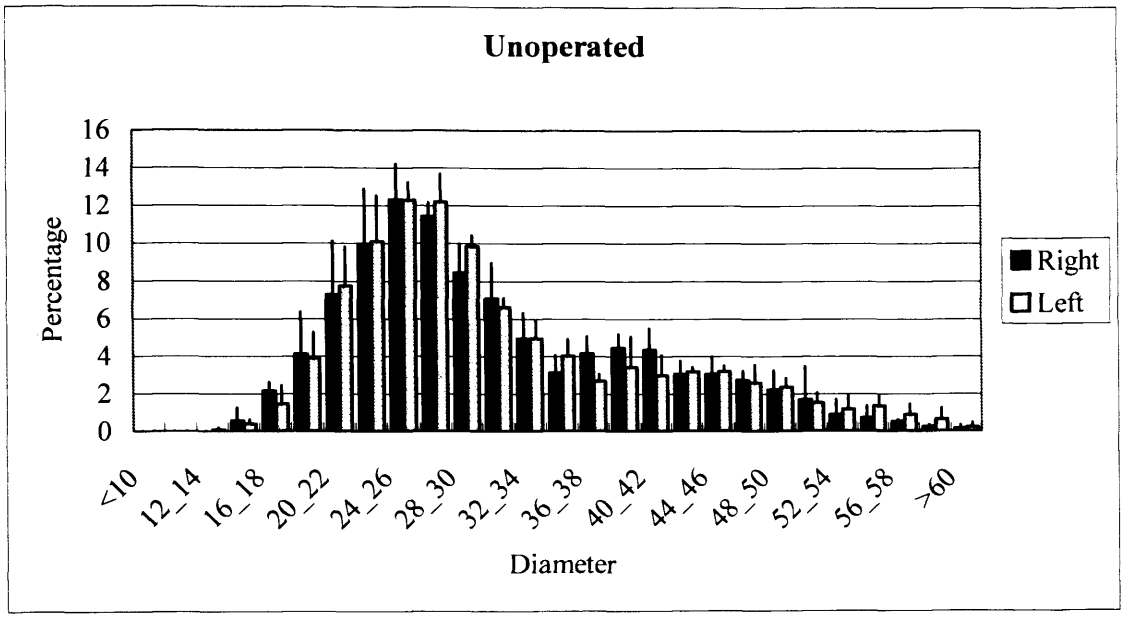


Figure 3.6A: Size frequency histogram (±SD) illustrating the distribution of ipsilateral (right) and contralateral (left) L4 & L5 DRG neurons in unoperated rats (Appendix 3).

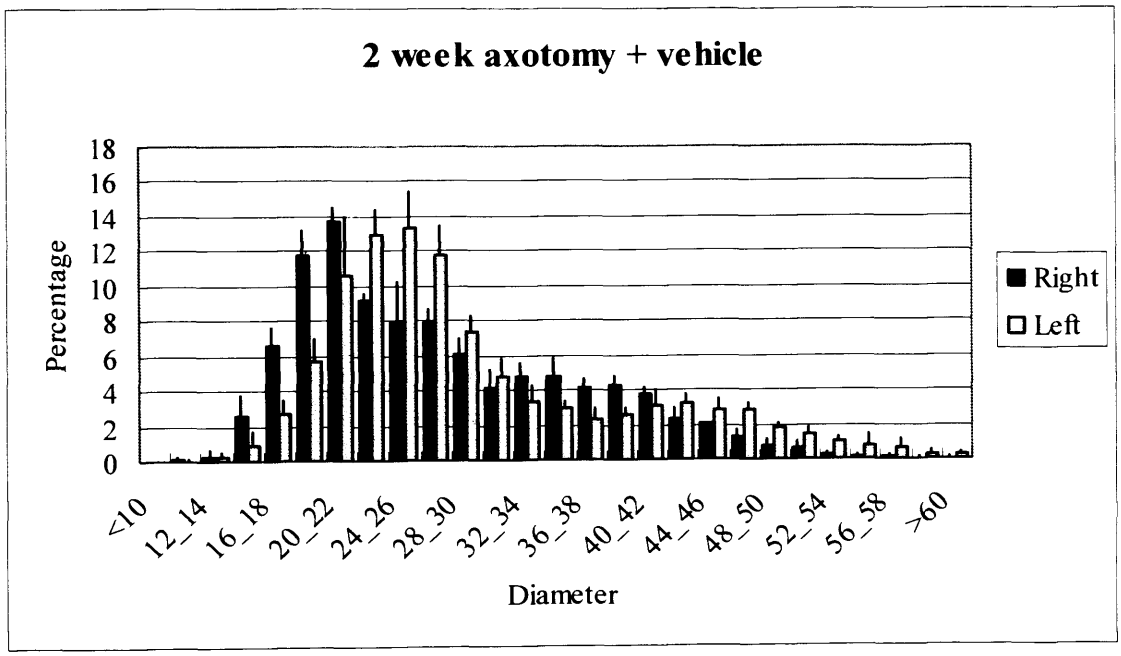


Figure 3.6B: Size frequency histogram (±SD) illustrating the distribution of ipsilateral (right) and contralateral (left) L4 & L5 DRG neurons after 2 week axotomy + vehicle administration (Appendix 4).

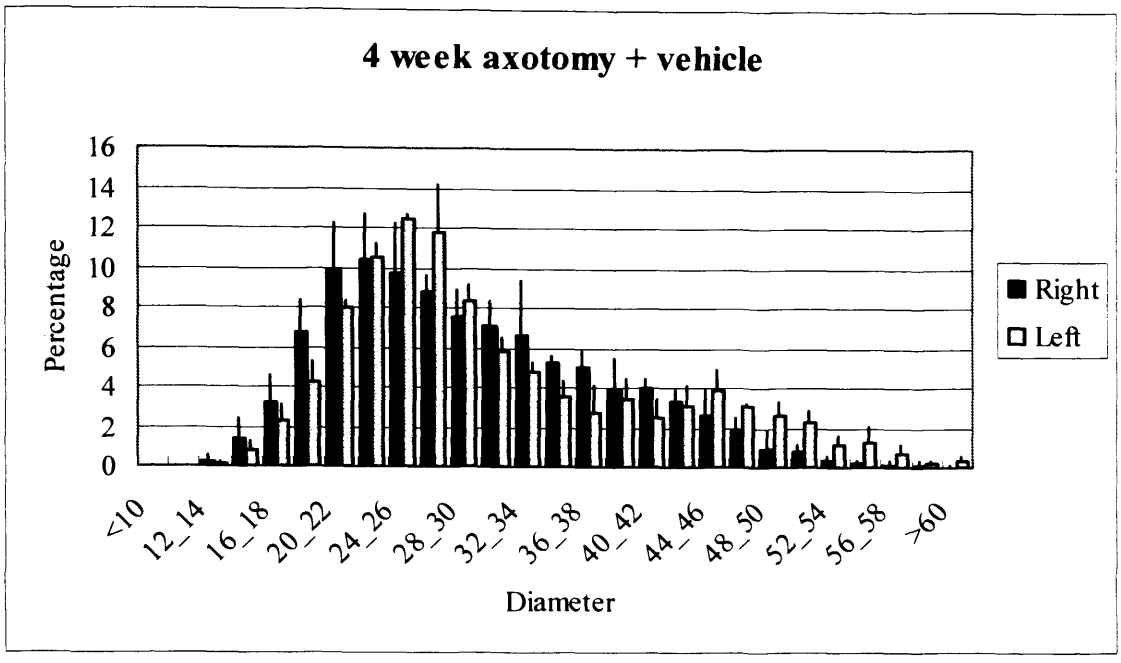


Figure 3.6C: Size frequency histogram (±SD) illustrating the distribution of ipsilateral (right) and contralateral (left) L4 & L5 DRG neurons after 4 week axotomy + vehicle administration (Appendix 5).

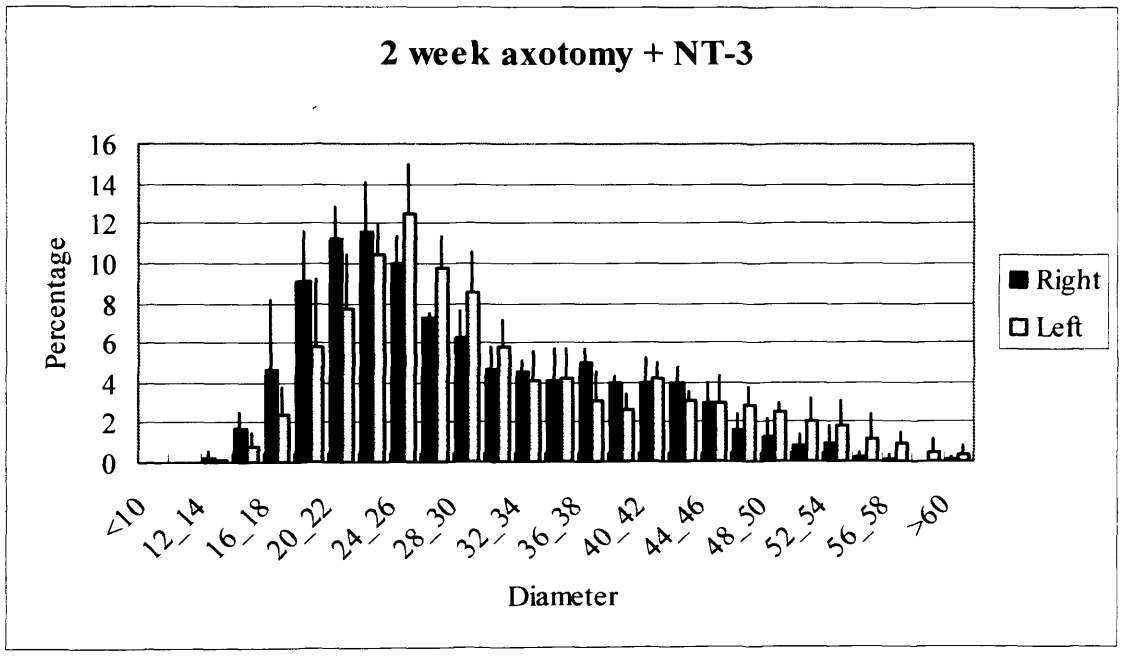


Figure 3.6D: Size frequency histogram (±SD) illustrating the distribution of ipsilateral (right) and contralateral (left) L4 & L5 DRG neurons after 2 week axotomy + NT-3 (1.25mg/4 weeks) administration (Appendix 6).

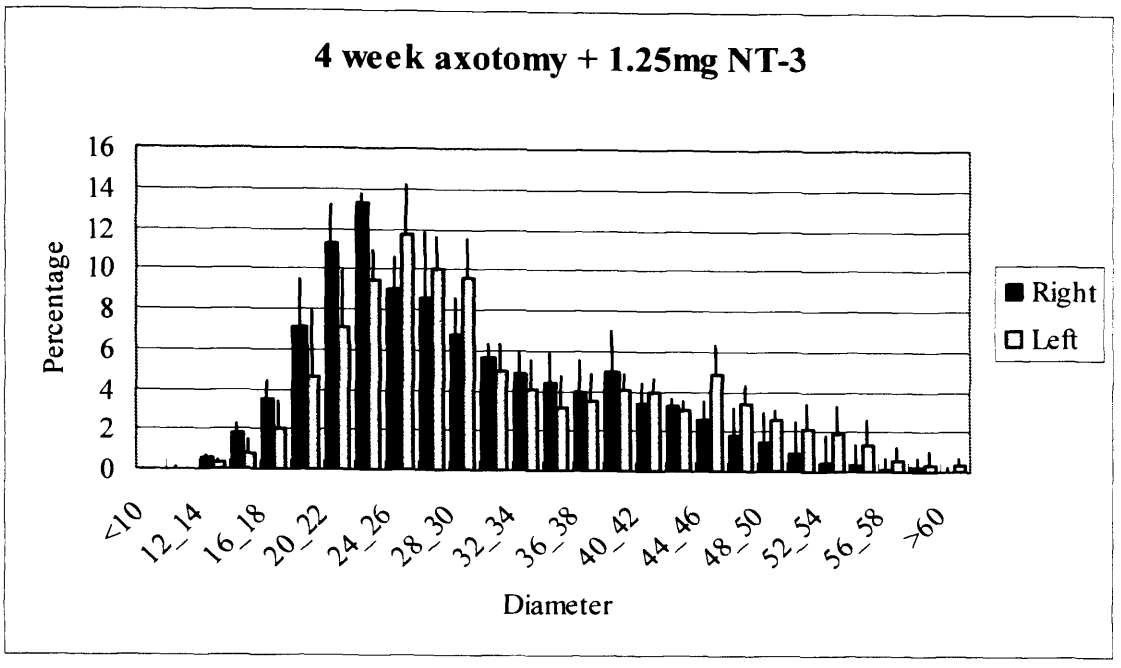


Figure 3.6E: Size frequency (±SD) histogram illustrating the distribution of ipsilateral (right) and contralateral (left) L4 & L5 DRG neurons after 4 week axotomy + NT-3 (1.25mg/4 weeks) administration (Appendix 7).

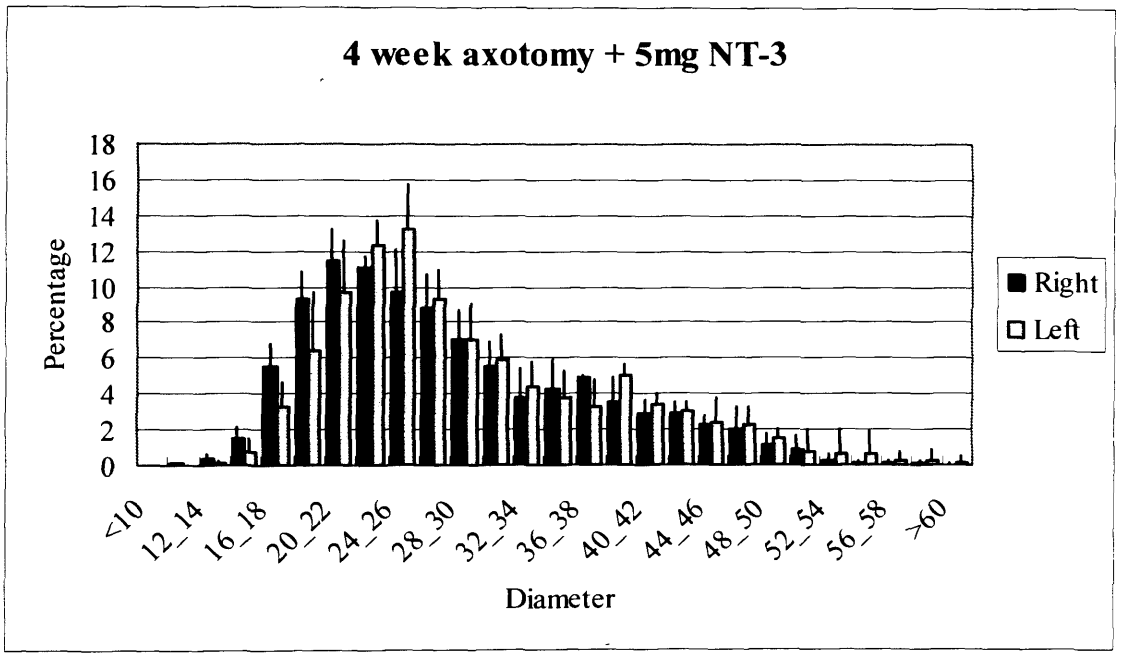


Figure 3.6F: Size frequency histogram (±SD) illustrating the distribution of ipsilateral (right) and contralateral (left) L4 & L5 DRG neurons after 4 week axotomy + NT-3 (5mg/4 weeks) administration (Appendix 8).

3.4 Effect of NT-3 on nestin mRNA and immunoreactivity in adult rat dorsal root ganglia

3.4.1 Axotomy-induced increase in nestin mRNA and immunoreactivity in adult rat DRG

I counted any neuron with light or strong nestin immunoreactivity distributed throughout its cytoplasm as positive. No neurons in normal or contralateral ganglia showed any nestin immunoreactivity at all, and the background staining was always minimal.

In normal L4 and L5 DRGs, nestin mRNA detected by PCR was expressed in normal rat DRG with low copy numbers and there was no difference between right and left DRGs (**Figure 3.7**). Some Schwann cells and satellite cells around large diameter neurons were nestin-immunoreactive (**Figure 3.8**), but there were no neurons showing nestin immunoreactivity in normal DRGs. At the time points I investigated, expression of mRNA for nestin in contralateral ganglia remained at the same levels after sciatic nerve transection.

In axotomised ganglia, nestin mRNA was markedly up-regulated at 1day and 2 weeks after operation (**Figure 3.7**). Although the mean expression at 4 weeks seemed even higher than at other times, this change was not significant, probably because of the relatively large variability from animal to animal in that group. Nestin immunoreactivity was observed in some cells with the size and morphology of small neurons after axotomy: 1.60% in ipsilateral ganglia 4 weeks following injury and 3.64% at 8 weeks (**Table 3.4**). The appearances of these nestin-immunoreactive cells varied from oval-shaped with peripherally-located nucleus (**Figure 3.8**) to round cells with a central nucleus. Immunoreactivity within these cells was concentrated around the nucleus and was usually

absent in the periphery of the neuron; no axons were seen to contain nestin-immunoreactivity.

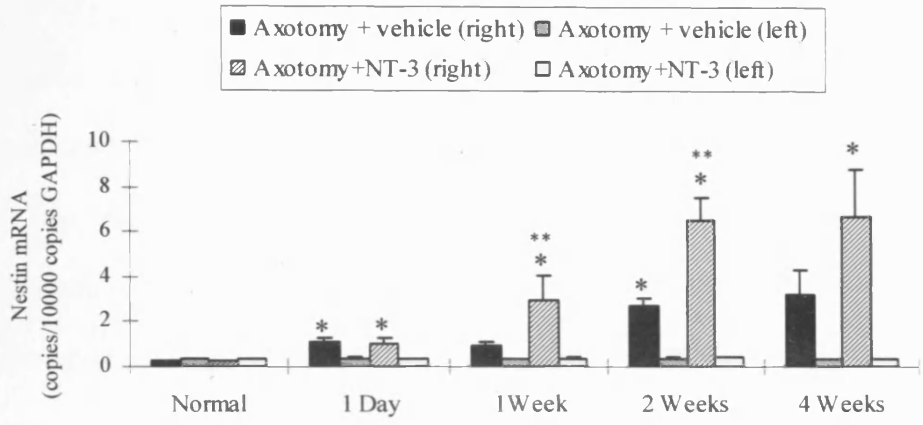


Figure 3.7: The expression of mRNA for nestin in L4 & L5 DRG after sciatic nerve transection with systemic vehicle or NT-3 administration (Mean ± S.E.M.). * indicates $p<0.05$ versus unoperated control of the same side DRG. ** indicates $p<0.05$ versus vehicle administration of the same side DRG (Appendix 10). ANOVA with Tukey's post hoc analysis.

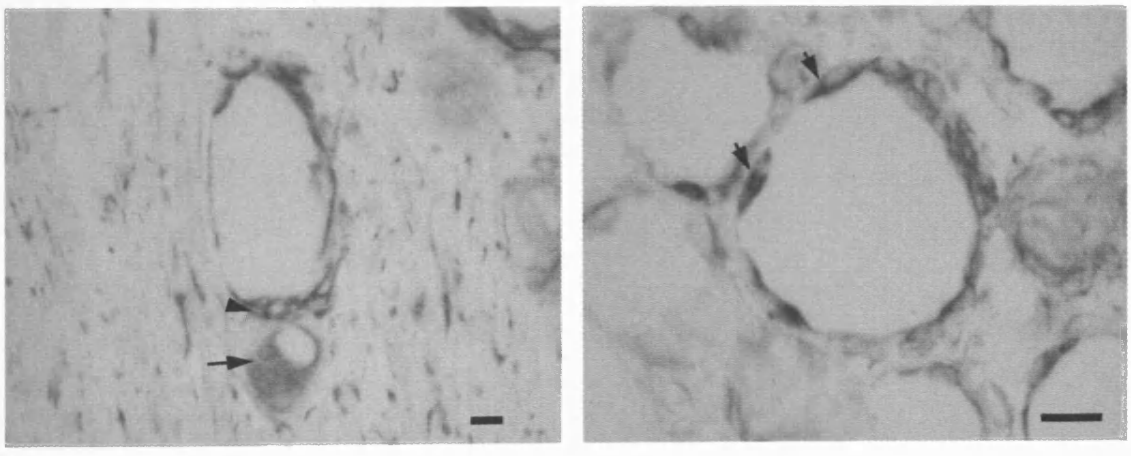


Figure 3.8: Immunohistochemistry shows nestin-immunoreactive (IR) neuron size cells (arrow) and satellite cells (arrowheads) in L4 & L5 DRG after sciatic nerve transection and 4 week systemic NT-3 administration (5mg). Scale bar = 10μm

3.4.2 Effect of NT-3 on mRNA expression and immunoreactivity for nestin

In axotomised DRG, the incidence of nestin-immunoreactive neurons was significantly up-regulated by NT-3 administration (5mg/4 weeks) 4 weeks following axotomy (Student's t test: $p<0.05$)(**Table 3.3**). Nestin mRNA expression in NT-3-treated ganglia was statistically significantly increased at 1 week and 2 weeks after axotomy when compared to vehicle-treated ganglia and elevated at 4 weeks when compared to unoperated animal (**Figure 3.6**). The intensity and pattern of immunostaining was similar to those in the vehicle-treated rats. These discrepancies between immunohistochemical and molecular data show that the effects of NT-3 administration on nestin mRNA expression may be mainly contributed by the nestin-immunoreactive non-neuronal cells in these DRGs.

Table 3.3: Estimated % of the nestin-immunoreactive neurons and nestin mRNA expression in ipsilateral (right) and contralateral (left) L4 & L5 DRGs in unoperated, 4 week sciatic nerve transection with vehicle or NT-3 administration and 8 week sciatic nerve transection with vehicle administration rats (Appendix 9).

Group	% of nestin-immunoreactive neurons in ipsilateral L4 & L5 (Mean ± SEM)	% of nestin-immunoreactive neurons in contralateral L4 & L5 (Mean ± SEM)	mRNA expression for nestin in ipsilateral L4 & L5 (copies/10000 copies of GAPDH; Mean ± SEM)
Unoperated rats (n=4)	0	0	0.28 ± 0.01
4 week sciatic transection with vehicle pump (n=4)	1.60 ± 0.46	0	3.20 ± 1.16
4 week sciatic transection with NT-3 (5mg/4 weeks) pump (n=4)	3.73 ± 1.28	0	6.66 ± 2.14
8 week sciatic transection with vehicle pump (n=4)	3.64 ± 1.21	0	--

3.4.3 Double immunofluorescence for nestin and β-III tubulin, trkA, trkC or CGRP

All of the nestin-immunoreactive neuron size cells seen in axotomised ganglia 4 and 8 weeks after axotomy with vehicle treatment and 4 weeks after axotomy with NT-3 treatment are also immunoreactive for β-III tubulin (**Figure 3.9**). Some of them express trkA (**Figure 3.10**), trkC (**Figure 3.11**) or CGRP (**Figure 3.12**). The characteristics and sizes of these cells were not different from those with only nestin immunoreactivity. Interestingly, nestin immunoreactivity is usually distributed in the center of neurons and is absent from the axon and the periphery of the perikaryon.

3.5 Effect of systemic NT-3 administration on mRNA expression of neurotrophins after axotomy

3.5.1 mRNA expression of neurotrophins in normal DRG

In this study, the mRNAs for NGF, BDNF and NT-3 were detected in DRGs from unoperated animals using both real-time quantitative PCR and *in situ* hybridisation (**Figure 3.13, 3.14A, 3.14D and 3.15A**), as reported by others [using *in situ* hybridisation, RT-PCR and northern blot analysis] (Sebert and Shooter, 1993; Zhou et al., 1999b). NGF mRNA was expressed in many small neurons (**Figure 3.14A**); BDNF mRNA was strongly expressed in some neurons of various sizes, especially small ones (**Figure 3.14D**); NT-3 mRNA was very weakly expressed in small and medium-sized neurons (**Figure 3.15A**). Satellite cells around large neurons were not observed to have any signal of these neurotrophins. From the results of real-time PCR (**Figure 3.13**), the much higher copy

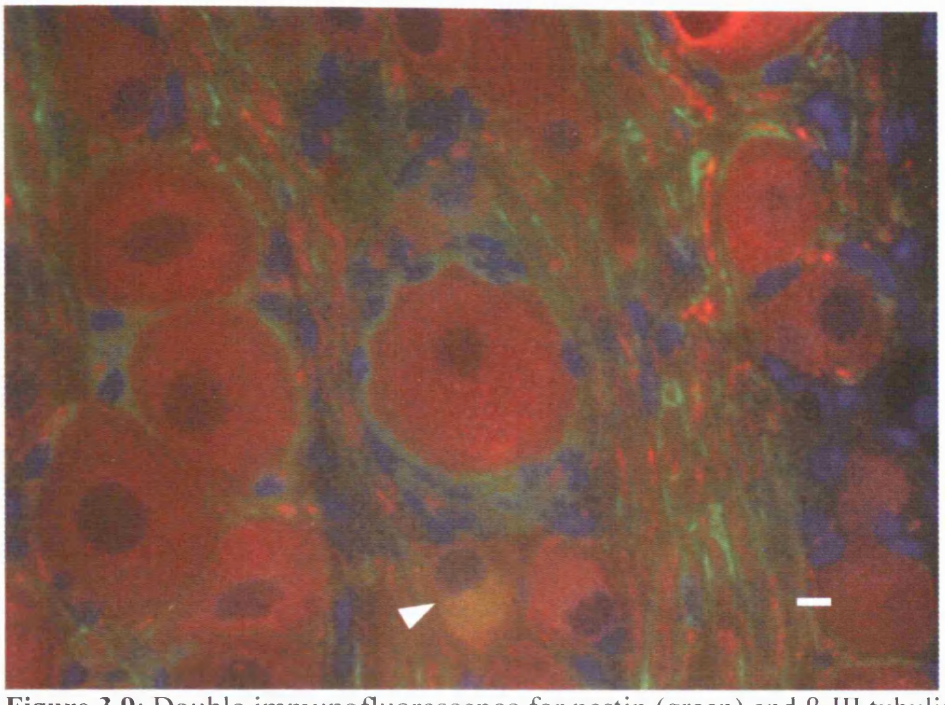


Figure 3.9: Double immunofluorescence for nestin (green) and β-III tubulin (red) shows a nestin- and β-III tubulin-IR cell (arrowhead) in L4 DRG after sciatic nerve transection and 8 week systemic vehicle administration. The blue colour is a nuclear fluorescent stain, DAPI (4',6-Diamidino-2-phenylindole dihydrochloride). Scale bar = 10μm

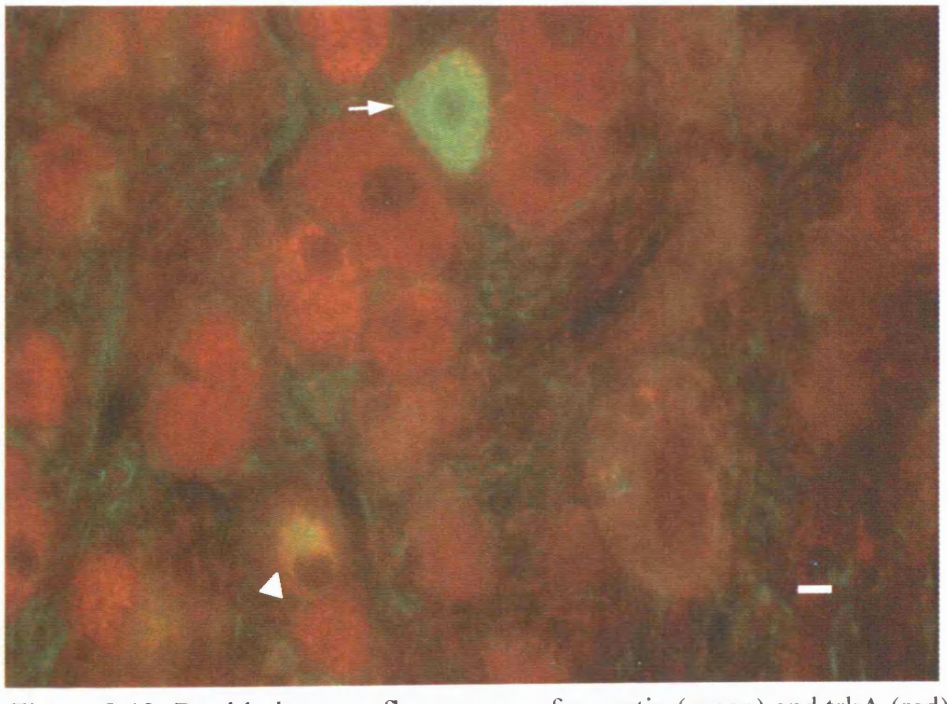


Figure 3.10: Double immunofluorescence for nestin (green) and trkA (red) shows a nestin- and trkA-IR cell (arrowhead) and a nestin-IR neuron (arrow) in L4 DRG after sciatic nerve transection and 8 week systemic vehicle administration. Scale bar = 10μm

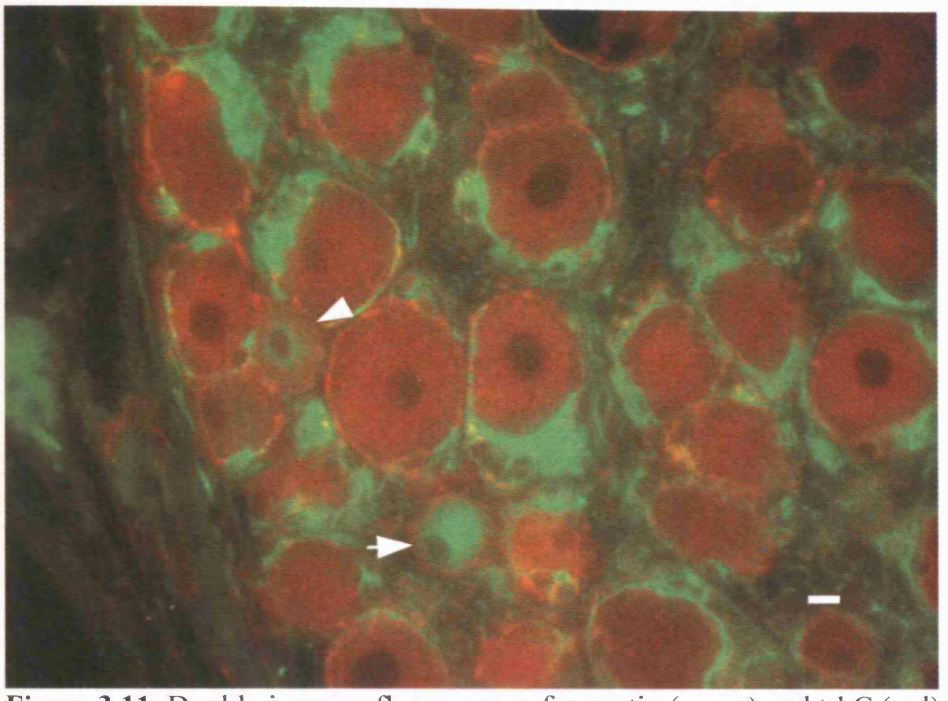


Figure 3.11: Double immunofluorescence for nestin (green) and trkC (red) shows a nestin- and trkC-IR cell (arrowhead) and a nestin-IR cell (arrow) in L4 DRG after sciatic nerve transection and 8 week systemic vehicle administration. Scale bar = 10μm

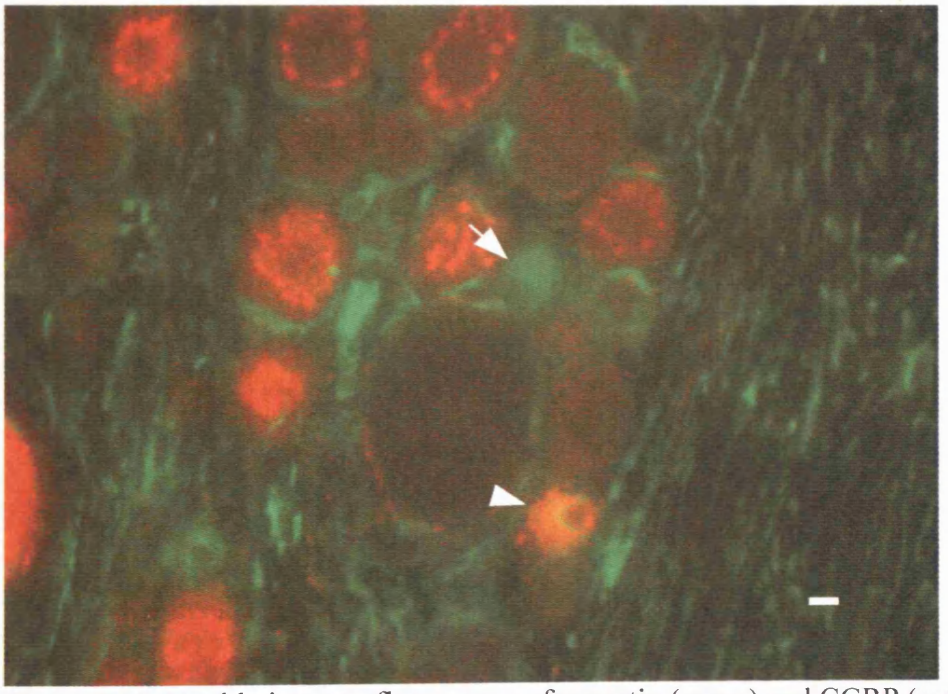


Figure 3.12: Double immunofluorescence for nestin (green) and CGRP (red) shows a nestin- and CGRP-IR cell (arrowhead) and a nestin-IR cell (arrow) in L4 DRG after sciatic nerve transection and 8 week systemic vehicle administration. Scale bar = 10μm

numbers of BDNF (around 140 copies/10000 copies GAPDH) than those for NGF (3.5 copies/10000 copies GAPDH) and NT-3 (12 copies/10000 copies GAPDH) implies that BDNF in uninjured DRG may play a more important role than the other two members of neurotrophin family. There was no significant difference ($p>0.05$) in levels of expression between the right and left DRGs in unoperated ganglia.

3.5.2 mRNA expression of neurotrophins in axotomised DRG

One day after sciatic transection, levels of NGF in the ipsilateral DRG increased by 4.7 fold and remained significantly higher than controls for at least one week ($p<0.05$; **Figure 3.13A**), largely in small-to-medium size neurons and some satellite cells as revealed by *in situ* hybridisation (**Figure 3.14B**).

BDNF expression increased by around 4 folds in small-to-large neurons at one day following axotomy and declined to control levels by one week (**Figure 3.13B and 3.14E**).

The expression of NT-3 mRNA had doubled at one day after axotomy ($p<0.05$; **Figure 3.13C**), mainly in small and some large neurons but also in satellite cells (**Figure 3.15B**), but showed no significant elevation by one week.

The contralateral ganglia showed no change of mRNA expression of all these neurotrophins in any vehicle-treated groups, except for a significant elevation for BDNF at one day ($p<0.005$) that had subsided to control levels by 1 week (**Figure 3.13B**).

3.5.3 Effect of NT-3 on neurotrophin expression in axotomised DRG

Systemic administration of NT-3 significantly reduced the increase in NGF mRNA level seen in the ipsilateral ganglia 1 day after sciatic transection ($p<0.05$), but significantly

increased it in medium-to-large neurons at 1 week compared with axotomised group ($p<0.05$; **Figure 3.13A and 3.14C**); by 4 weeks there was no significant difference in ipsilateral NGF mRNA levels between NT-3- and vehicle-treated groups. These data suggested that the exogenous NT-3 prolonged the elevation in NGF mRNA seen after axotomy for at least 2 weeks. The NGF mRNA in contralateral ganglia remained unaffected by NT-3 administration.

The expression of BDNF mRNA in the ipsilateral ganglia of NT-3-treated rats was significantly increased at all time points, expressed in most small neurons and more medium-to-large neurons, compared with those from vehicle-treated rats (**Figure 3.13B and 3.14F**). However, NT-3 administration prevented the elevation in BDNF mRNA seen at one day in the contralateral ganglia in vehicle-treated animals.

The profile of NT-3 mRNA expression was not significantly altered in ipsilateral or contralateral ganglia by NT-3 administration when compared to vehicle-treated rats, mainly in small neurons (**Figure 3.13C and 3.15C**).

Overall, these results suggest that the exogenous NT-3 has stimulated or maintained the expression of mRNAs for NGF and BDNF, at least temporarily.

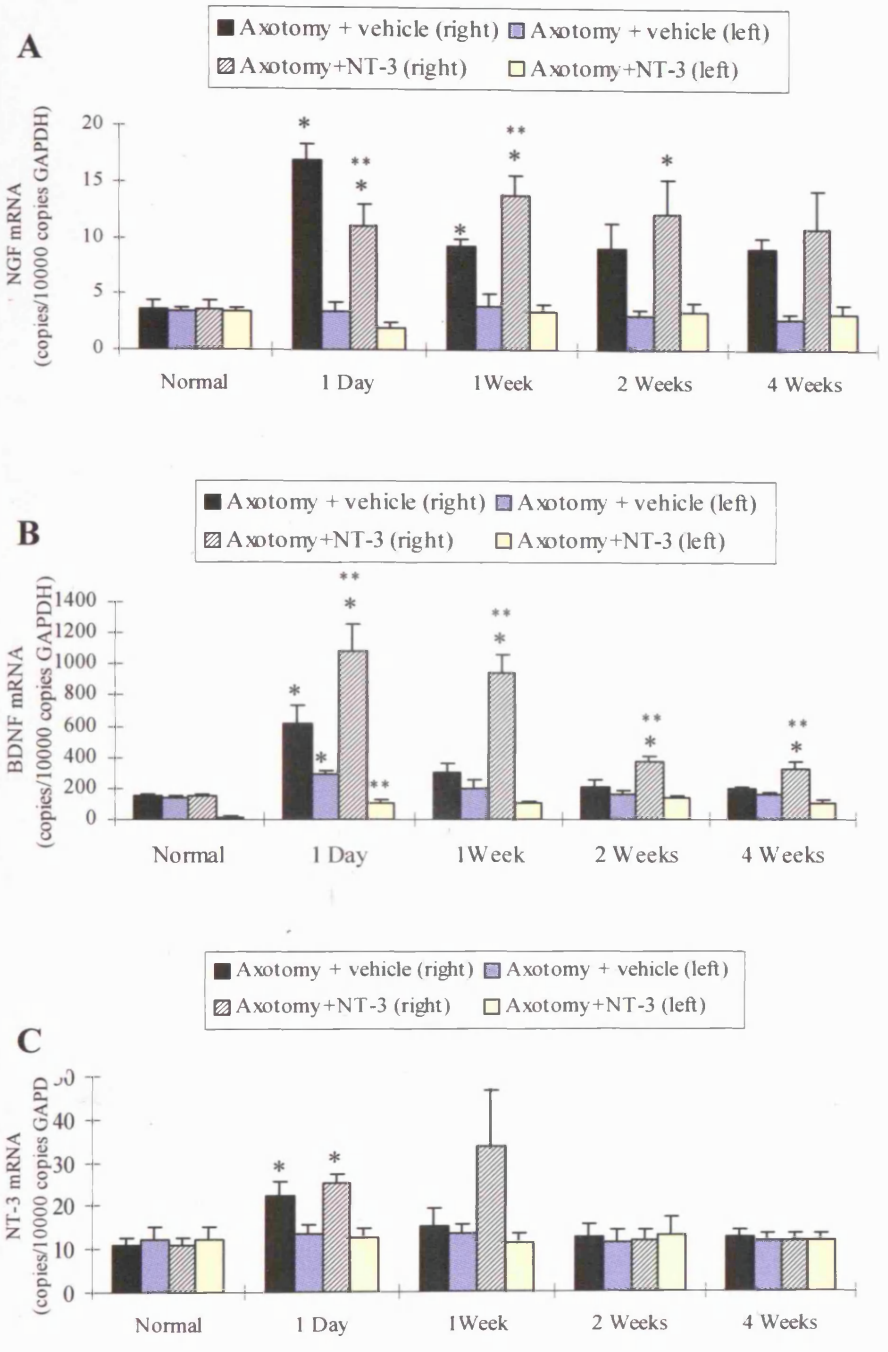


Figure 3.13: Neurotrophin expression. NGF (A), BDNF (B) and NT-3 (C) mRNA expression in L4 and L5 DRG after sciatic transection with systemic NT-3 or vehicle administration (Mean ± S.E.M.). * indicates $p<0.05$ versus unoperated control of same side DRG. ** indicates $p<0.05$ versus vehicle administration of the same side DRG; ANOVA with Tukey's post hoc analysis (Appendix 11, 12 and 13).

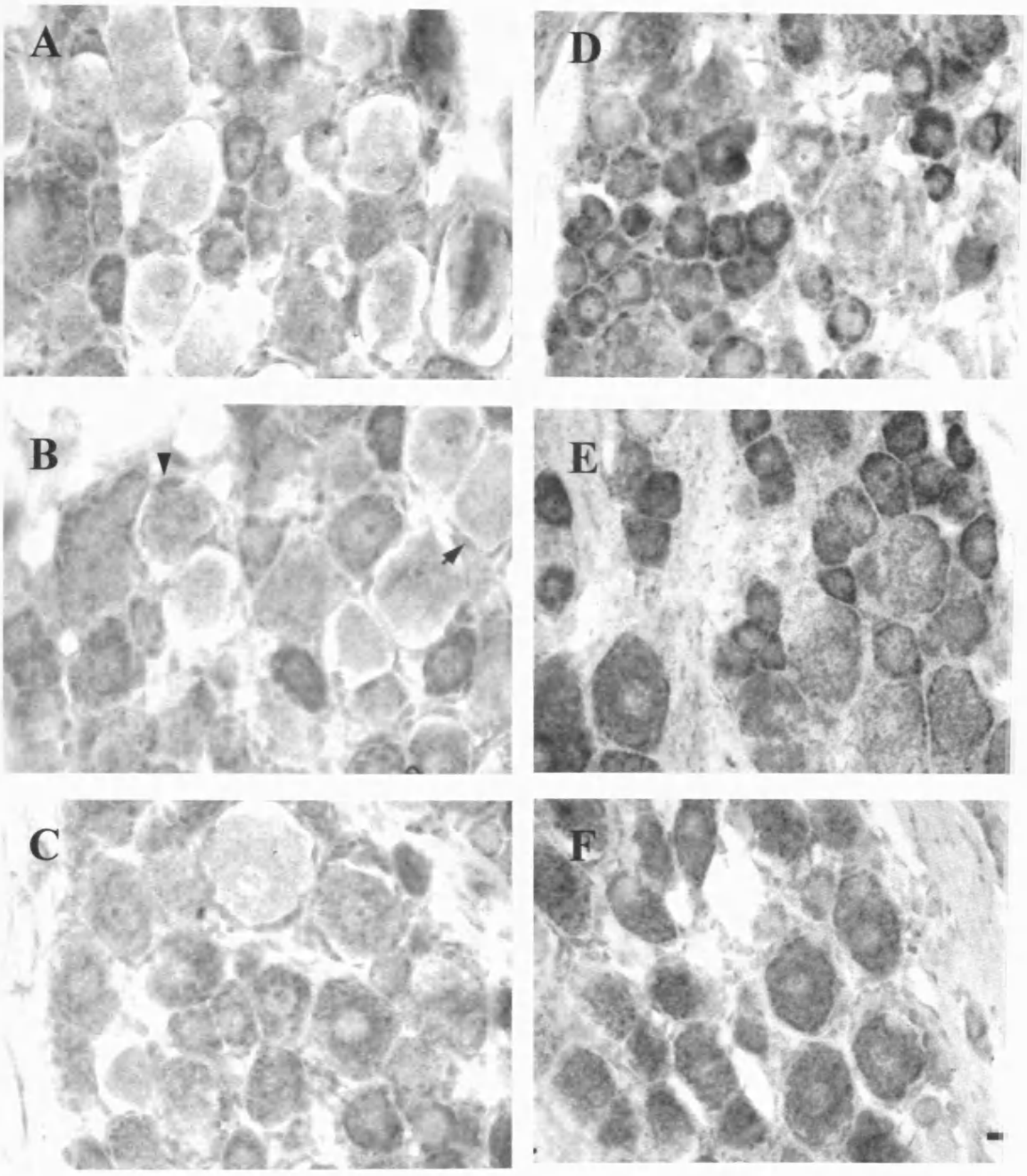


Figure 3.14: *In situ* hybridisation for NGF mRNA in unoperated rat lumbar DRG (A), o week after axotomy with vehicle (B) and NT-3 (C) treatment in ipsilateral ganglia; BDN mRNA in unoperated DRG (D), 1 day after axotomy with vehicle (E) and NT-3 (F) treat t in ipsilateral ganglia. Scale bar = 10μm. Arrowheads indicate mRNA expression in satel cells.

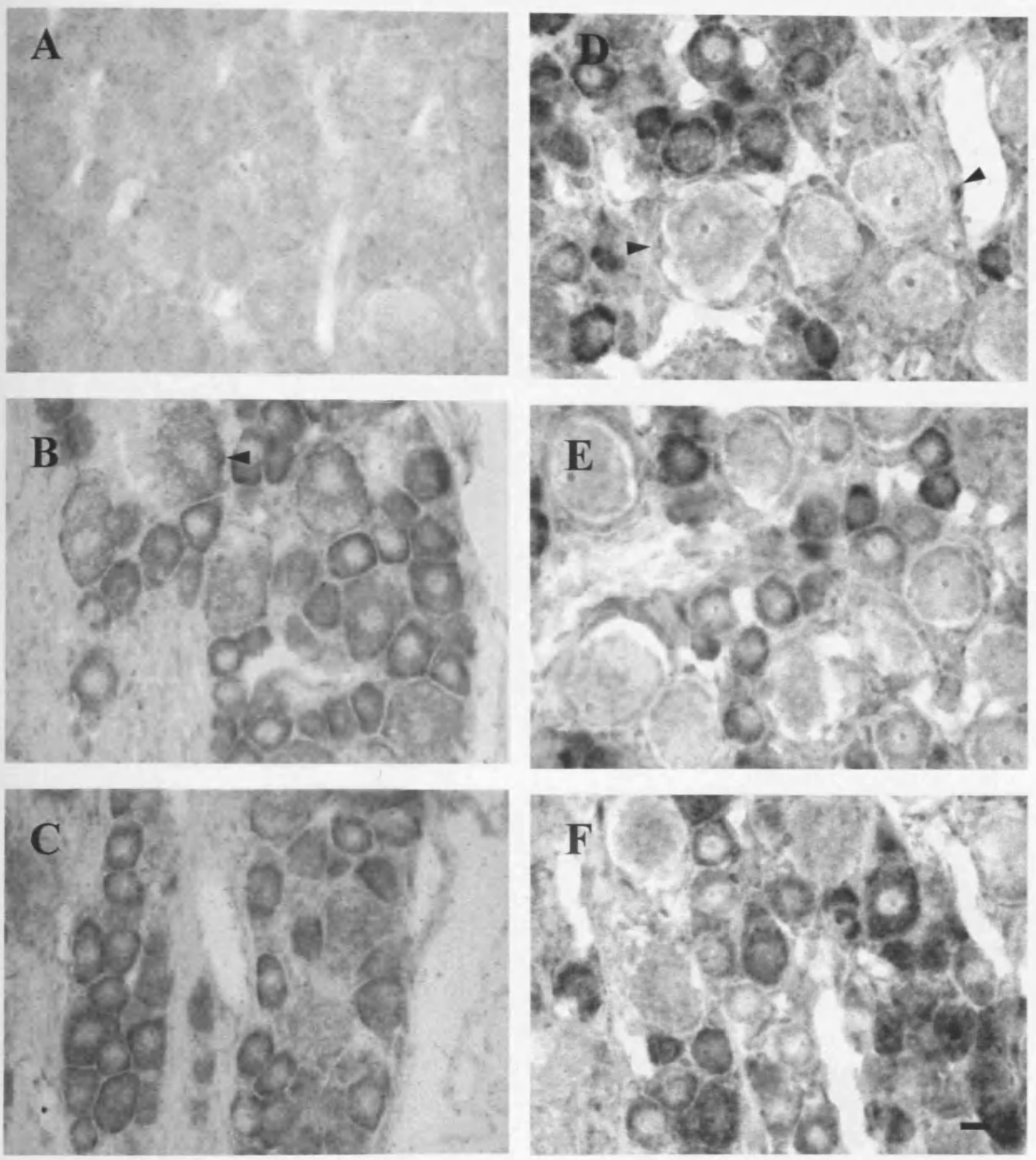


Figure 3.15: *In situ* hybridisation for NT-3 mRNA in unoperated rat lumbar DRG (A), 1 day after axotomy with vehicle (B) and NT-3 (C) treatment in ipsilateral ganglia; trkA mRNA in unoperated DRG (D), 2 weeks after axotomy with vehicle (E) and NT-3 (F) treatment in ipsilateral ganglia. Scale bar = 10μm. Arrowheads indicate mRNA expression in satellite cells.

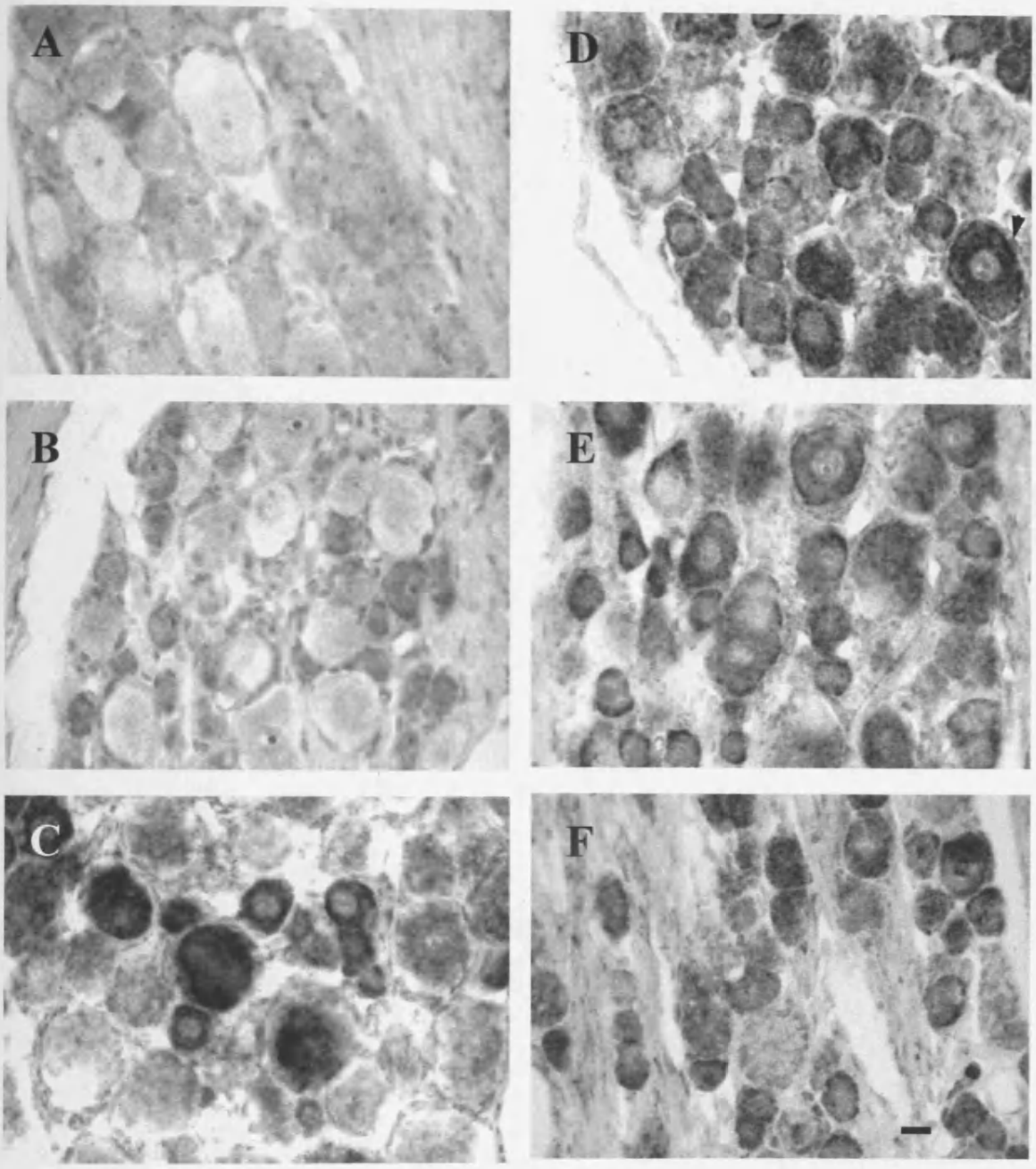


Figure 3.16: *In situ* hybridisation for trkB mRNA in unoperated rat lumbar DRG (A), 2 week after axotomy with vehicle (B) and NT-3 (C) treatment in ipsilateral ganglia; trkC mRNA in unoperated DRG (D), one day after axotomy with vehicle treatment (E) and 2 weeks after axotomy with NT-3 treatment (F) in ipsilateral ganglia. Scale bar = 10μm. Arrowheads indicate mRNA expression in satellite cells.

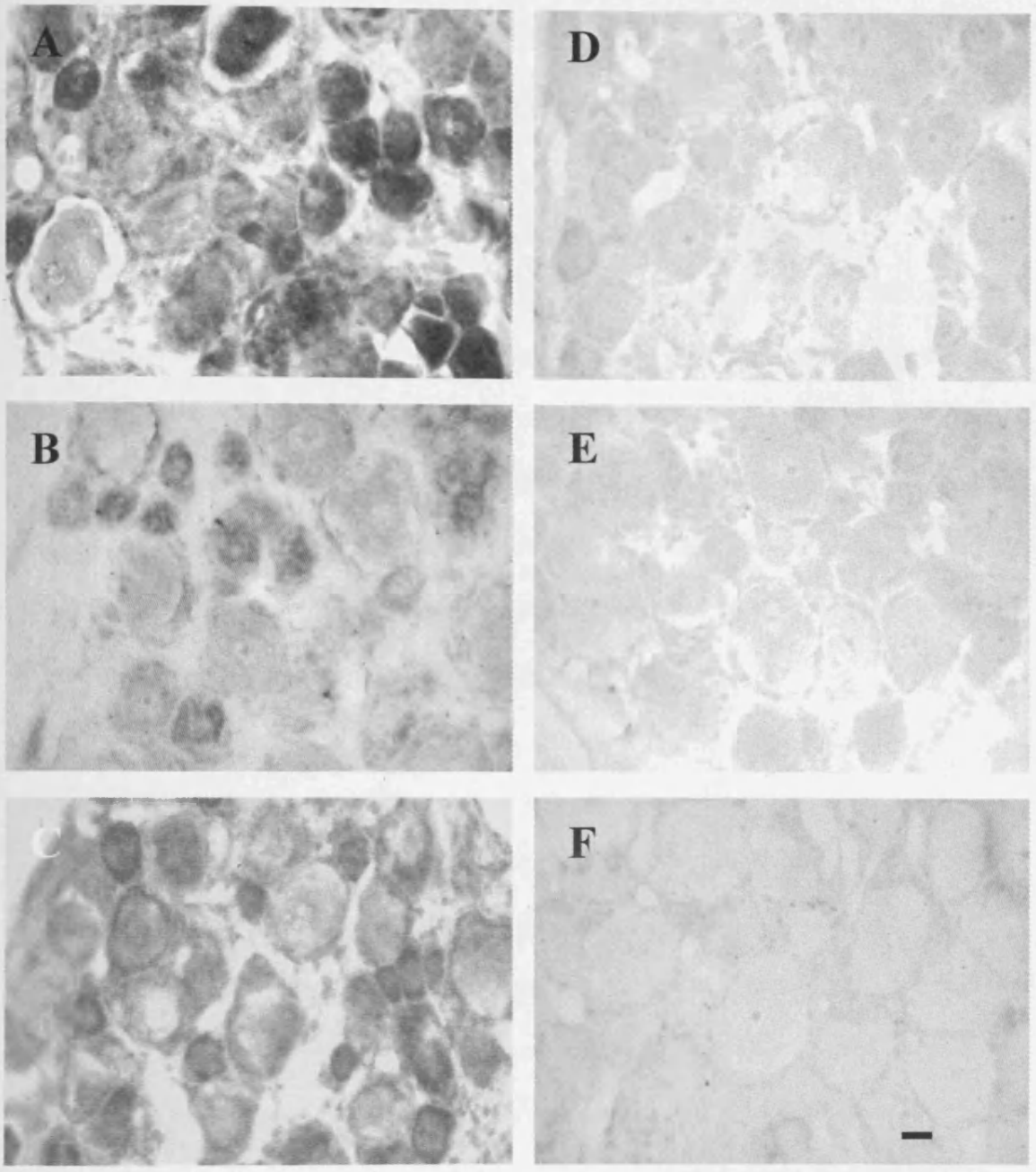


Figure 3.17: *In situ* hybridisation for $p75^{NTR}$ mRNA in unoperated rat lumbar DRG (A), 1 week after axotomy with vehicle in ipsilateral DRG (B) and 2 weeks after axotomy with NT-3 treatment in contralateral DRG (C); negative control using sense probe for NT-3 mRNA (D), trkA mRNA (E) and trkC mRNA (F). Scale bar = 10μm.

3.6 Effect of systemic NT-3 administration on mRNA expression of neurotrophin receptors after axotomy

3.6.1 Expression of neurotrophin receptors in normal DRG

The mRNAs of all neurotrophin receptors were detectable in normal DRG by real-time quantitative PCR and *in situ* hybridisation. In terms of copy number and localisation, trkA showed the highest expression in small neurons (**Figure 3.15D and 3.18A**) and trkB showed the lowest copy numbers in various size neurons (**Figure 3.16A and 3.18B**); TrkC was expressed in medium-to-large neurons (**Figure 3.16D and 3.18C**), and all size neurons for $p75^{NTR}$ (**Figure 3.17A and 3.18D**). Occasionally satellite cells also showed trkA and trkC mRNA expression (**Figure 3.15D and 3.16D**).

3.6.2 Expression of neurotrophin receptors in axotomised DRG

Following sciatic transection, the level of mRNA for trkA in ipsilateral ganglia was reduced by ~55% at two weeks ($p<0.005$; **Figure 3.18A**), after which it recovered to control levels. *In situ* hybridisation showed that trkA mRNA expression was still located in small neurons (**Figure 3.15E**). However, in contralateral ganglia the level of trkA mRNA was significantly elevated 1 day after sciatic nerve transection in neurons and satellite cells, compared with unoperated control levels. The error bar (SEM) shows the small variation of the expression of this gene between animals.

The level of trkB mRNA was significantly decreased by 2 weeks after transection, when its expression was 30% of unoperated control levels ($p<0.05$; **Figure 3.16B and**

3.18B), returning to control levels by 4 weeks. The levels in contralateral ganglia did not alter significantly at any time point.

The levels of trkC mRNA were, in contrast to trkA and trkB, dramatically elevated in ipsilateral ganglia following sciatic transection, peaking at one day when the level was over six times that in unoperated ganglia ($p<0.01$; **Figure 3.18C**). *In situ* hybridisation showed that the increase was mainly in neurons of all sizes (**Figure 3.16E**), although some satellite cells also expressed trkC mRNA, particularly those associated with large neurons. The levels then decreased slowly but still remained significantly higher than normal until 4 weeks after axotomy, by which time they had returned to normal levels. In the contralateral ganglia the trkC mRNA level was significantly increased at 1 day ($p<0.005$) and 4 weeks ($p<0.05$), in all sizes of neuron, but not in satellite cells.

The amount of mRNA encoding the low-affinity neurotrophin receptor $p75^{NTR}$ in DRGs of both sides quickly decreased to a level below 10% of control values one day following sciatic nerve transection, and was only expressed in some small neurons ($p<0.05$; **Figure 3.17B and 3.18D**). The level remained depressed on both sides for at least one week and did not return to unoperated control levels until 2 weeks.

3.6.3 Effect of NT-3 on neurotrophin receptor expression in axotomised DRG

NT-3 administration produced no effect on trkA and trkB mRNA expression in ipsilateral ganglia at one day and one week after injury compared to vehicle-treated rats (**Figure 3.18**). However, at 2 weeks both trkA and trkB mRNA expression was up-regulated by NT-3 administration in some neurons of all sizes, compared with vehicle-treated animals ($p<0.005$ and $p<0.001$ respectively; **Figure 3.15F, 3.16C, 3.18A and**

3.18B), and at 4 weeks it was increased by 1.9- and 3.9-fold when compared with unoperated control levels ($p<0.005$). In contralateral DRGs from NT-3-treated animals the increase in trkA mRNA expression was prevented at 1 day after axotomy. Conversely, the expression of trkB mRNA in contralateral DRGs was significantly increased at 1 week in neurons, and by 4 weeks the level of trkB expression increased to the same level as in the ipsilateral ganglia.

The mRNA for trkC remained at unoperated control levels in ipsilateral ganglia one day and one week after sciatic transection and NT-3 treatment, but at 2 weeks had significantly increased when compared to unoperated controls ($p<0.001$, **Figure 3.16F and 3.18C**), although it was still significantly lower than in ipsilateral ganglia from vehicle-treated animals ($p<0.05$). *In situ* hybridisation showed that at 2 weeks trkC mRNA was expressed in all sizes of neuron, especially small-to-medium neurons. By 4 weeks trkC mRNA levels in ipsilateral ganglia from NT-3-treated animals were elevated three-fold over unoperated control levels ($p<0.005$), and were significantly higher than that in ipsilateral ganglia from vehicle-treated animals ($p<0.05$). NT-3 administration significantly increased trkC mRNA expression in contralateral ganglia at 4 weeks only.

$p75^{NTR}$ transcripts decreased markedly after axotomy, but NT-3 administration had no effect, compared with vehicle-treated ganglia, on the profile of $p75^{NTR}$ mRNA expression in ipsilateral ganglia after sciatic transection (**Figure 3.18D**). However, in complete contrast with the change in ipsilateral ganglia, the $p75^{NTR}$ mRNA level in contralateral ganglia was significantly increased by NT-3 treatment at all time points, and at 4 weeks was around 2.5-fold higher when compared with unoperated control ganglia. *In*

situ hybridisation showed that the increased expression appeared to be due to up-regulation in small neurons and satellite cells (**Figure 3.17C**).

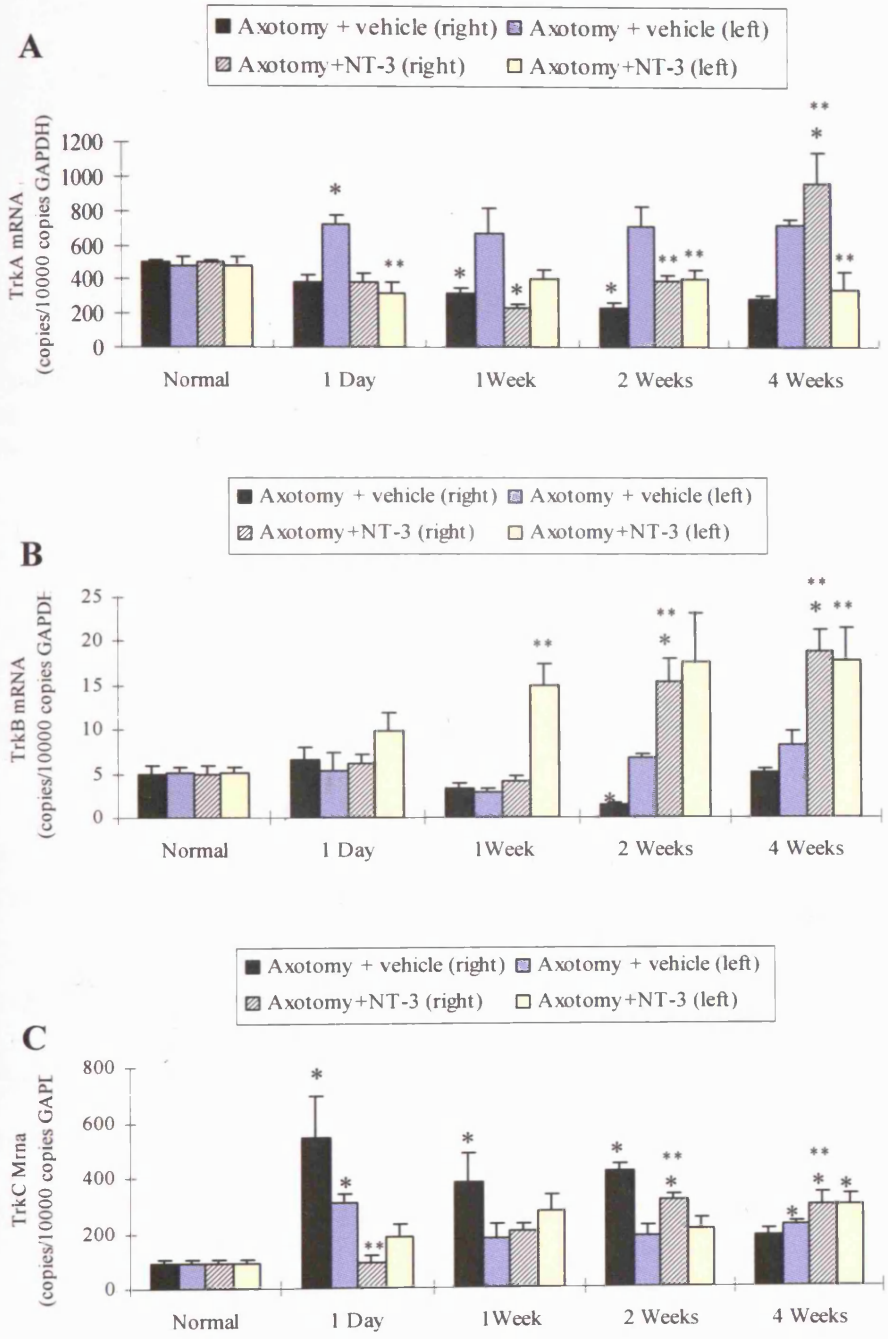


Figure 3.18 (for legend see next page)

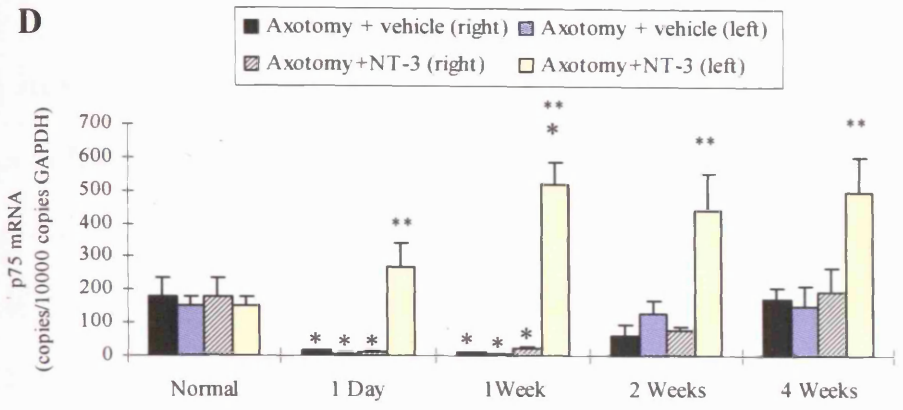


Figure 3.18: Neurotrophin receptor expression. The expression of mRNA for TrkA (A), TrkB (B), TrkC (C) and $p75^{NTR}$ (D) in rat L4 and L5 DRG after sciatic transection with systemic NT-3 or vehicle administration (Mean ± S.E.M.). * indicates $p<0.05$ versus unoperated control of same side DRG. ** indicates $p<0.05$ versus vehicle administration of the same side DRG; ANOVA with Tukey's post hoc analysis (Appendix 14, 15, 16 and 17).

3.7 Gene expression profile in adult rat dorsal root ganglion after axotomy and systemic NT-3 administration

3.7.1 Quality of array hybridisation

A cDNA library was analysed to identify changes in gene expression resulting from axotomy and subsequently systemic NT-3 administration; L4 and L5 DRG tissue from adult rats was collected 2 weeks after right sciatic nerve transection and ligation with either systemic vehicle or NT-3 administration, as well as from unoperated rats. To check the quality of total RNA extracted from these ganglia before carrying out further steps of microarray analysis, quality of RNA samples were assessed by capillary electrophoresis on Bioanalyzer 2100 Agilent to ensure the 28S : 18S rRNA ratio was > 1.0 for each sample; an example was shown in **Figure 3.19**. All the nine RNA samples passed the requirements.

For each group (unoperated, axotomy + systemic vehicle administration and axotomy + systemic NT-3 administration), three chips were used. Twelve rats were used for each group. After target hybridisation and image scanning, a few parameters, such as noise factor (Q), scaling and normalisation factors, intensity of background and the percentage of present genes in each array, were listed to show the quality of hybridisation (**Table 3.4**). Noise factor is a measure of the pixel-to-pixel variation of probe cells on an array. Electrical noise of the scanner leads to a significant portion of Q value. To make it possible to compare data from different arrays, average intensities of gene expression were normalised to a same value of 100 using a scaling factor before further analysis. Larger discrepancies among scaling factors (greater than three-fold) indicate significant assay variability or sample degradation. All scaling factors in this study were within a two fold change. The percentage of genes present in each array ranged from 27 to 39% of the genes included.

A few controls were used, including B2 oligo arranged on the boundaries of the probe area to as a positive control, bioB, bioC, bioD and cre as hybridisation controls to evaluate sample hybridisation efficiency to gene expression arrays, and actin and GAPDH as internal control genes to check degradation by the ratio of the 3' probe set to the 5' probe set.

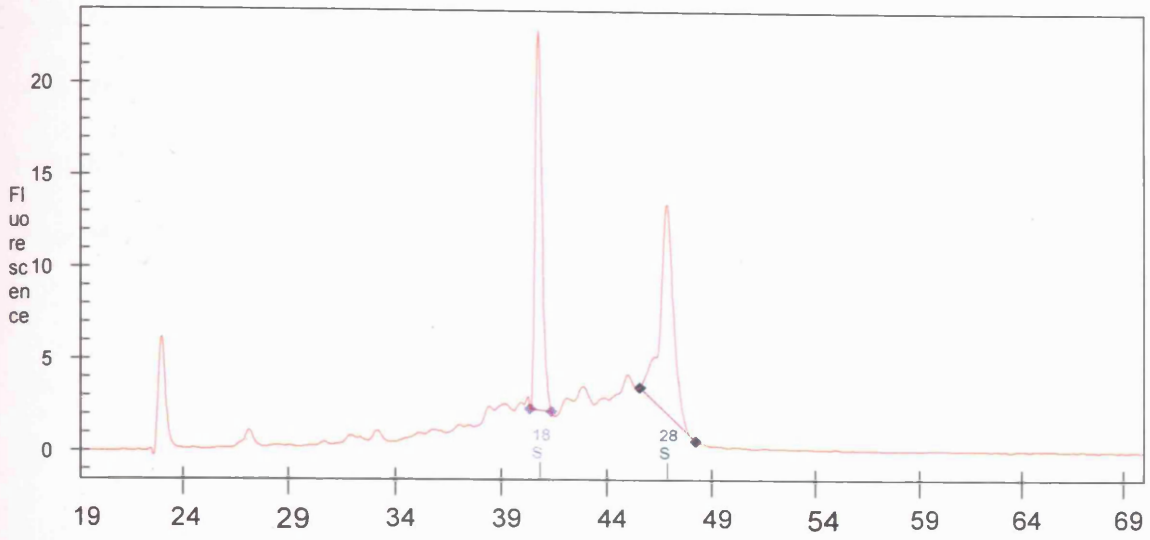


Figure 3.19: RNA quality was assessed by capillary electrophoresis to ensure the 28S : 18S rRNA ratio was > 1.0 for each sample.

Table 3.4: Details of microarray hybridisation.

Chip No	Procedure	Q (noise factor)	Scaling Factor	Back-ground	SD of background	Genes present (%)
1	Unoperated	1.73	1.27	55.42	0.89	32.10
2		3.51	1.91	134.27	4.66	31.70
3		1.97	0.56	65.9	1.37	39.30
4	Axotomy + vehicle	1.58	1.09	49.29	0.67	35.30
5		1.75	0.64	56.67	0.98	38.60
6		3.05	0.86	115.93	6.3	29.10
7	Axotomy + NT-3	1.62	1.02	51.84	0.8	33.20
8		2.83	1.73	89.34	4.05	27.50
9		3.28	0.91	124.56	7.28	27.00

SD: Standard deviation

3.7.2 Gene expression profile in adult rat DRG after axotomy

The gene expression profile in vehicle-treated animals was quantitatively compared with unoperated controls and NT-3-treated animals to find out the genes regulated by axotomy and by systemic NT-3 treatment 2 weeks after injury. Three chips were used for

each group and cross-comparisons were made between all the chips to generate nine pair-wise comparisons between unoperated and axotomy + vehicle rats and nine pair-wise comparisons between axotomy + vehicle and axotomy + NT-3 rats. The fold changes are presented as mean ± standard deviation of the nine pair-wise comparisons.

In addition to the genes already functionally known, the cDNA microarrays used in this study include EST sequences. The technology of sequencing expressed sequence tag (EST), which is is a short sub-sequence of a protein-coding DNA sequence, offers a relatively cheap alternative to whole genome sequencing and has become a valuable resource for gene discovery (Lindlof, 2003). Based on the concept that proteins participating in similar biological pathways often have similar expression profiles (Hughes et al., 2000), the use of a cDNA microarray that includes EST sequences has made it possible to obtain interesting functional insights from the temporal expression profiles of newly detected genes. To analyse the regulated EST functionally might lead to the discovery of novel genes that contribute to nerve regeneration. Of the 1322 known genes and ESTs represented on the Rat neurobiology U34 array, 357 (27.0%) genes were consistently detected in all arrays. Based on the potential functions of the genes, they were categorised into 9 classes (**Table 3.5**).

Figure 3.20 A, B and C shows a correlation of the signal intensity values of all the expressed transcripts between two unoperated chips, two axotomy + vehicle chips and two axotomy + NT-3 chips. The signal intensity observed for each expressed transcript was very similar between the three chips in each group. The correlation coefficients between any two chips in each group were 0.895-0.964 (mean: 0.932) in unoperated group, 0.967-0.993 (mean: 0.979) in axotomy + vehicle group, and 0.970-0.983 (mean: 0.975) in

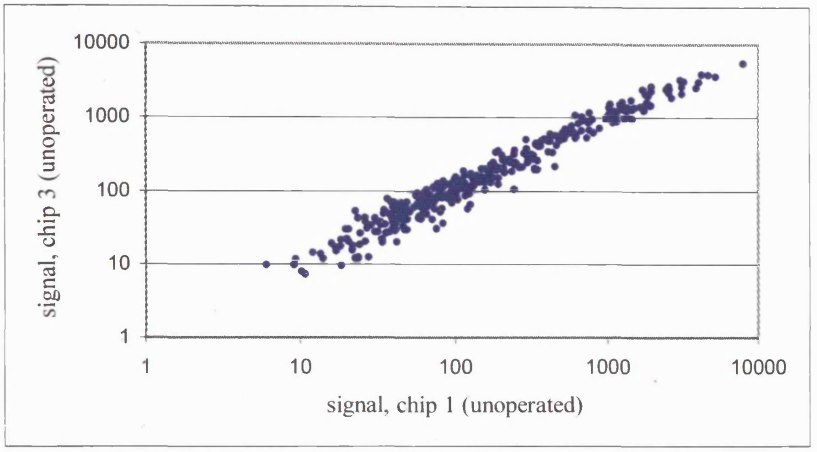
axotomy + NT-3 group, which confirmed the reproducibility of the three triplicate chips in each group.

1.5-fold change in signal intensity was used as a cut-off line to consider the differential expression of a gene as significant. Among those genes and ESTs, 69 showed an increase in expression greater than 1.5-fold, and 49 a decrease in expression greater than 1.5-fold when comparing DRG at 2 weeks after axotomy with DRGs from the unoperated control animals (**Figure 3.21A**). Twenty-nine genes and ESTs increased more than 1.5-fold and 15 genes and ESTs decreased more than 1.5-fold in DRGs from NT-3-treated animals compared with those from vehicle-treated animals (**Figure 3.21B**). The coefficient of variance (CV= standard deviation/mean) is the degree to which a set of data points varies, which is used to to assess the *precision* of a technique. When assessing precision, the lower the coefficient of variance percentage, the better the precision between replicates. Among the regulated genes, the CV for 147 of these was less than 0.5; for 14 genes the CV was between 0.5 and 1.0, indicating good reproducibility of the three arrays in each group. To verify the array results, quantitative real-time PCR was performed to confirm a total of 7 genes (NF-L, NP-Y, 5HT-3R, $p75^{NTR}$, HSP70, peripheral-type benzodiazepine receptor, glutamate receptor subnit 5-2) and show that our microarray results accurately reflect the molecular changes (**Table 3.6, Figure 3.22**).

The full names and the GenBank accession numbers of the transcripts observed to change more than 1.5 fold between unoperated and vehicle-treated samples are given in **Tables 3.5A and B**. The genes regulated by systemic NT-3 by more than 1.5 fold are categorised in **Table 3.5C and D**. There is good correlation between some of the genes in the present study and others' previous observations (Costigan et al., 2002; Xiao et al.,

2002), substantiating the accuracy of our data. The functional implications of the change in genes regulated by axotomy and NT-3 administration will be discussed in **Discussion**.

A



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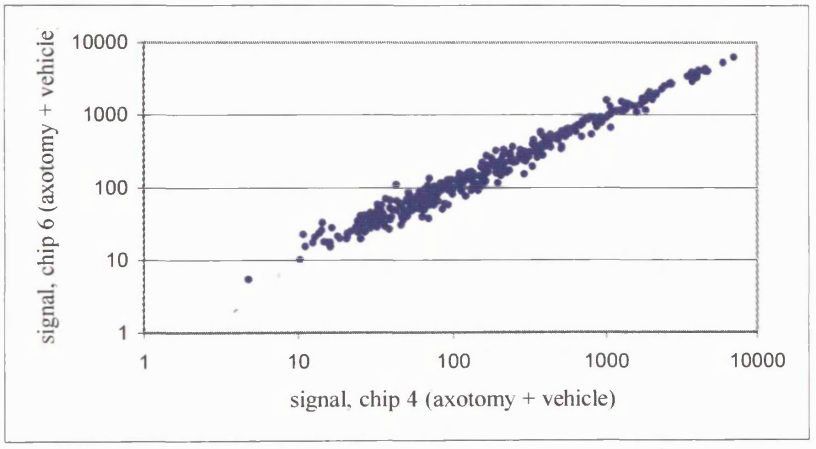

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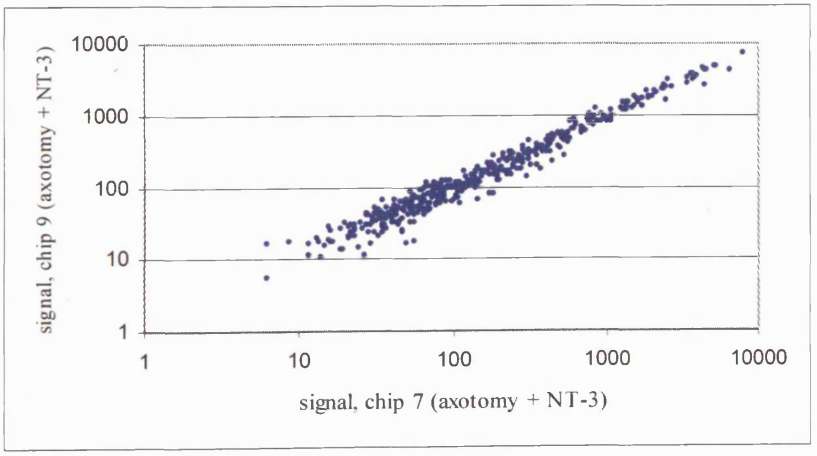

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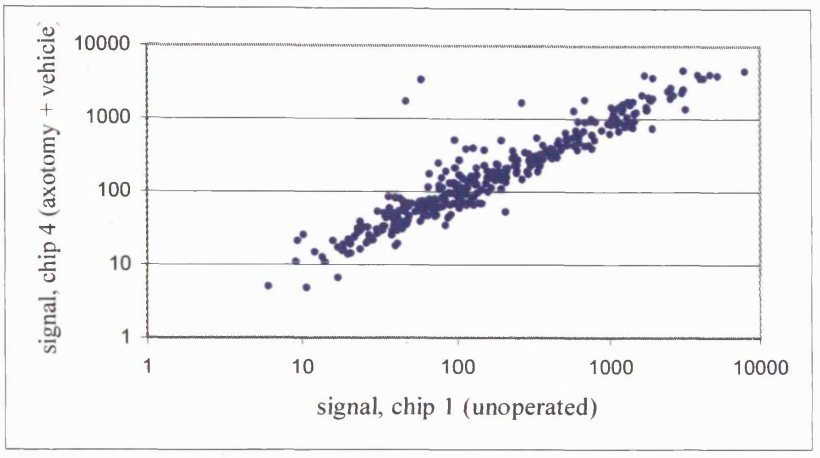

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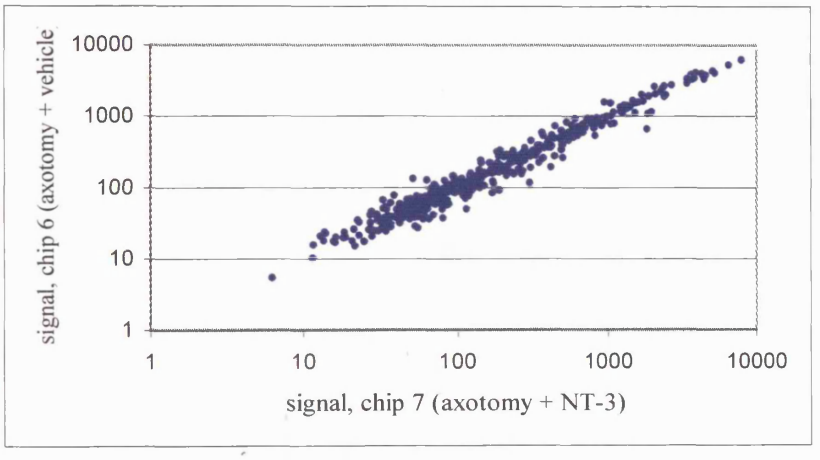

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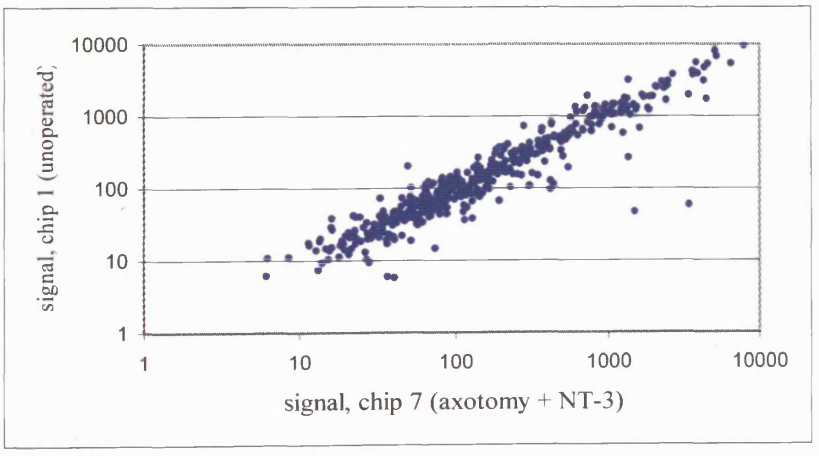


Figure 3.20: The correlation of the hybridisation signal intensity for all the expressed genes between two unoperated chips (A), two axotomy + vehicle chips (B), two axotomy + NT-3 chips (C), an axotomy + vehicle chip and an unoperated chip (D), an axotomy + vehicle chip and an axotomy + NT-3 chip (E), and an unoperated chip and an axotomy + vehicle chips (F).

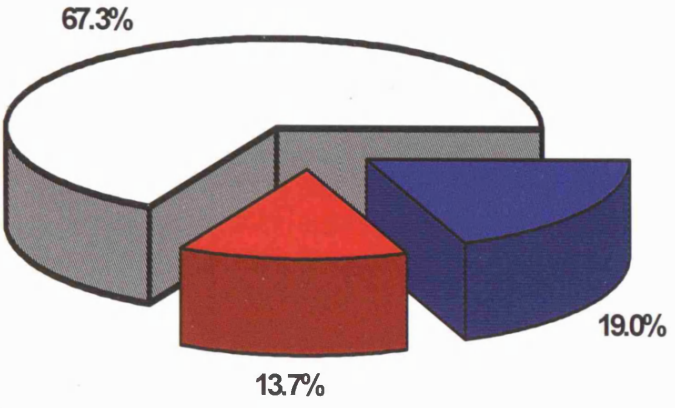


Figure 3.21A: Percentage of regulated genes 2 weeks after axotomy + systemic vehicle administration (compared with unoperated). "Blue colour" indicates up-regulation; "red colour" indicates down-regulation; "white colour" indicates genes detected but not changed.

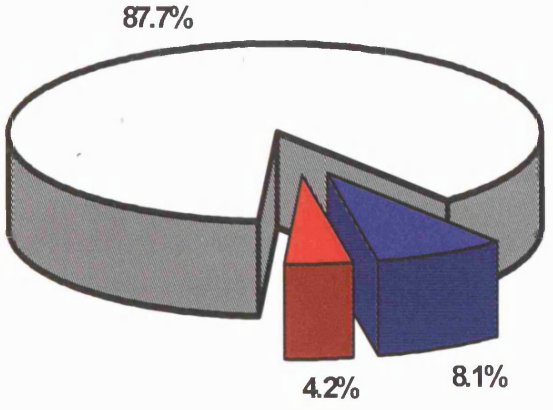


Figure 3.21B: Percentage of regulated genes 2 weeks after axotomy + systemic NT-3 administration (compared with axotomy + vehicle administration). "Blue colour" indicates up-regulation; "red colour" indicates down-regulation; "white colour" indicates genes detected but not changed.

Table 3.5A: Genes, grouped according to functional activity, whose expression was increased following 2 week sciatic nerve transection and vehicle administration, compared to unoperated animal (Appendix 18).

Group	Gene name (Fold change, mean)
Signal transduction and regulation gene expression	Mothers against dpp 1 homolog (Mad1) (4.50); Interferon-gamma inducing factor isoform alpha precursor (IGIF) (3.44); VGF (3.44); Immediate-early serum-responsive JE gene (MCP-1) (2.82); Cytosolic retinol-binding protein (CRBP)(1.88); Transferrin (1.70); Guanine nucleotide binding protein beta 1 subunit (1.57); Microtubule associated protein (MAP2c)(1.56); Janus protein tyrosine kinase 1 (JAK1)(1.55); G protein beta1 subunit (rGb1) (1.55); Ras GTPase-activating protein (1.52)
Pain-related genes	Neuropeptide Y(NP-Y)(54.53); Galanin (25.79); Corticotropin releasing factor (CRF)(1.92); P2X2-3 receptor (P2X2)(1.51)
Receptor and membrane protein	Peripheral-type benzodiazepine receptor (PBS)(4.31); MRC OX-45 surface antigen (2.19); Neuronal high affinity glutamate transporter (2.06); Glutamate/aspartate transporter protein (1.95); GABA-A receptor α-5 subunit (1.95)
Growth-associated protein and growth factors	GAP-43 (2.40); Basic fibroblast growth factor (2.00); Neurotrophin-3 (NT-3)(1.97); Brain-derived neurotrophic factor (BDNF)(1.65); Insulin-like growth factor binding protein (rIGFBP-6)(1.63); Insulin-like growth factor II (1.57)
Ion transport	Dihydropyridine-sesitive L-type calcium channel alpha-2 subunit (CCHL2A)(5.19); Brain sodium channel III (1.98); Neuronatin alpha (1.93); Neuron specific calcium-binding protein hippocalcin (1.87)
Secreted and extracellular molecules	Synaptic vesicle protein (SV2)(2.28); Synaptotagmin IV homolog (1.73); Syntaxin B (1.53); Synaptotagmin associated 35kDa protein (1.52)
Cytoskeleton	Glial fibrillary acidic protein alpha (GFAP)(3.45); Microtubule-associated protein (MAP) 1B (2.94); Cytoplasmic beta-actin (1.95); RB109 (brain specific protein)(1.73); Mu-calpain large subunit (cls1)(1.45)
Apoptosis	Calmodulin-dependent protein kinase II-delta (2.27)

Others	Heat shock protein (Hsp27)(2.97); Monoamine oxidase A (2.68); Manganese-containing superoxide dismutase (MnSoD)(2.43); A rat novel protein which is expressed with nerve injury (1.70); Tissue-type plasminogen activator (t-PA)(1.65); Neural adhesion molecule F3 (1.55)

Table 3.5B: Genes, grouped according to functional activity, whose expression was decreased following 2 week sciatic nerve transection and vehicle administration, compared to unoperated animal (Appendix 19).

Group	Gene name (Fold change, mean)
Signal transduction and regulation gene expression	Noggin (-1.89); GTP-binding protein (G-alpha-0)(-1.78); Phosphodiesterase I (-1.71); Presenilin-2 (-1.54); Ca2+/calmodulin-dependent protein kinase IV kinase isoform (-1.51)
Pain-related genes	Beta-tachykinin (-2.03); Beta-type calcitonin gene-related peptide (CGRP)(-1.80); RET ligand 2 (RETL2)(GDNFR)(-1.79); substance P precursor (-1.60); Somatostatin-14 (-1.60); Nicotinic acetylcholine receptor alpha 3 subunit (-1.57); Nicotinic acetylcholine receptor subunit beta4 (-1.54); Somatostatin (-1.52)
Receptor and membrane protein	Glutamate receptor subunit 5-2 (GluR5-2)(-2.13); Neural membrane protein 35 (-1.86); ET-B endothelin receptor (-1.83); Fast nerve growth factor receptor ($p75^{NTR}$)(-1.69); Transferrin receptor (-1.65); Meotropic glutamate receptor (GLUR4)(-1.63); GABA-B receptor (-1.60)
Ion transport	Potassium channel, alpha subunit (Kv9.3)(-3.81); CLC-5 chloride channel (-2.62); Voltage-gated Na channel alpha subunit NaN (-2.48); Potassium channel (Kv2.2; CDRK)(-2.16); Voltage-gated sodium channel (SNS)(-2.00); Na,K-ATPase alpha-1 subunit (-1.91); 5HT3 receptor subunit (-1.85); 5HT3 receptor (-1.85); Sodium channel I (-1.77); Potassium channel, alpha subunit (Kv9.1)(-1.71); Na+,K+-ATPase beta-3 subunit (-1.55); Putative chloride channel (-1.55); Putative potassium channel TWIK (-1.54); S100 alpha (-1.52); Potassium channel protein (3145 bp)(-1.51)

Secreted and extracellular molecules	Synaptosomal-associated protein 25 (SNAP-25A)(-1.73); Ca2+/calmodulin-dependent protein kinase II isoform gamma-b (CAMK2)(-1.61); Rat ras-related mRNA rab3 (-1.55)
Cytoskeleton	Neurofilament protein-light chain (NF-L)(-2.12); Microtubule-associated protein 1A (MAP1A)(-1.55); Heavy neurofilament polypeptide NF-H C-terminus (-1.52)
Others	Neurodegeneration associated protein 1 (-1.53)

Table 3.5C: Genes, grouped according to functional activity, whose expression was increased following 2 week sciatic nerve transection and NT-3 administration, compared to vehicle-treated animals (Appendix 20).

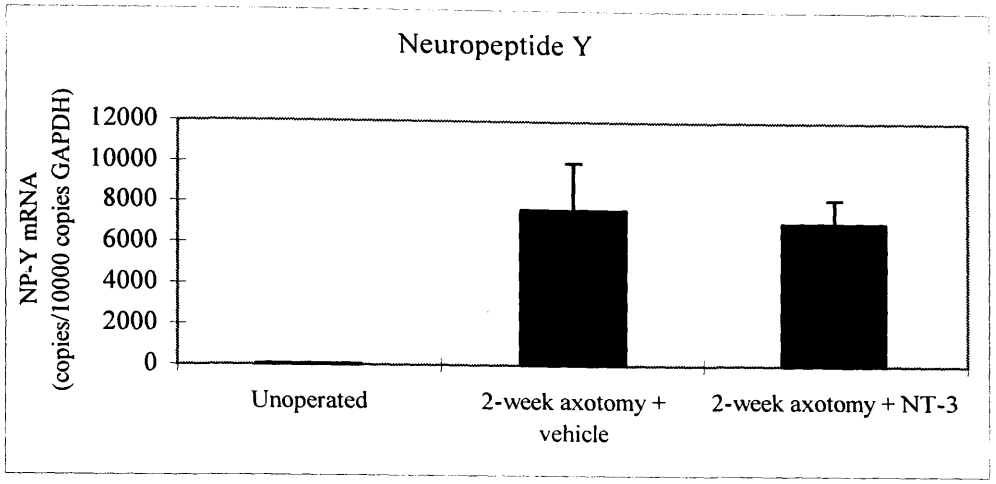
Group	Gene name (Fold change, mean)
Signal transduction and regulation gene expression	Phosphoinositide 3-kinase regulatory subunit p85alpha (2.99); Neuron-specific cortexin (1.97); cAMP response element binding protein (CREB)(1.67); Mothers against dpp 1 homolog (Mad1)(1.52); GTP-binding protein (G-alpha-0)(1.50); Chemokine CX3C (1.50)
Pain-related genes	Major hippocampal somatostatin receptor (SSTR4)(1.69); Calcitonin receptor-like receptor (CRLR)(1.67); Muscarinic receptor m2 (1.67)
Receptor and membrane protein	AMPA-selective glutamate receptor-A (1.92); ET-B endothelin receptor (1.50)
Growth-associated protein and growth factors	Fibroblast growth factor (FGF-18)(1.72)
Ion transport	Potassium channel (1.73); Calcium channel alpha-1S subunit (ROB1)(1.51)
Secreted and extracellular molecules	Syntaxin 4 (1.71)
Cytoskeleton	Cytoplasmic beta-actin (1.67); Glial fibrillary acidic protein alpha (GFAP)(1.54)
Others	Phosphoneuroprotein 14 (1.57); Calnexin (1.55); Olfactory receptor-like protein (SCR D-8)(1.55)

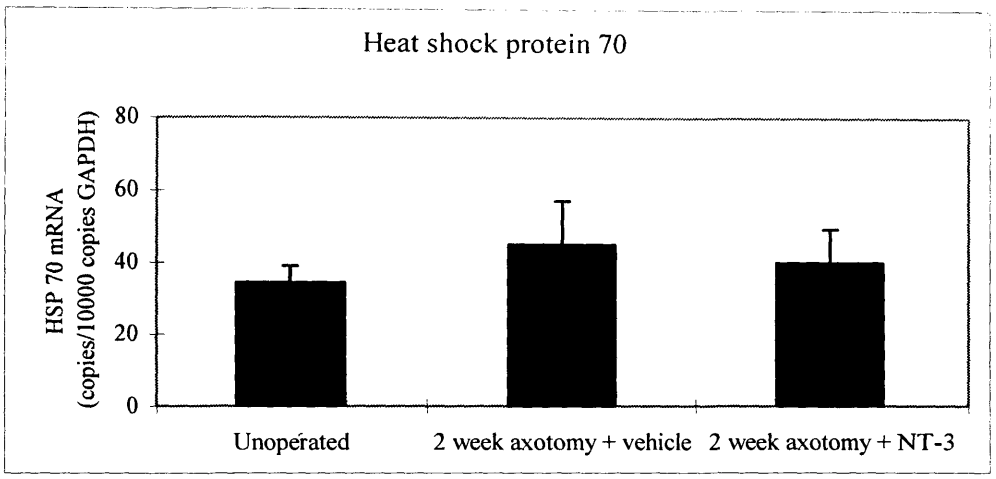
Table 3.5D: Genes, grouped according to functional activity, whose expression was decreased following 2 week sciatic nerve transection and NT-3 treatment, compared to vehicle-treated animals (Appendix 21).

Group	Gene name (Fold change, mean)
Signal transduction and regulation gene expression	Rat immediate-early serum-responsive JE gene (MCP-1)(-1.58); G protein beta1 subunit (rGb1)(-1.58)
Receptor and membrane protein	Beta-arrestin 2 (-1.50)
Ion transport	Sodium/potassium ATPase alpha-1 subunit truncated isoform (-1.95); Voltage-dependent sodium channel alpha subunit [SS1-SS2 segment, transmembrane segments IVS3-IVS6](-1.56)
Secreted and extracellular molecules	SNAP-25B (-1.68); Synaptojanin (-1.59); Rat ras-related mRNA rab3 (-1.58); Synapsin Ia (-1.54); SNAP-25A (-1.53)
Cytoskeleton	Microtubule-associated protein 1B (-2.53)
Others	Tissue-type plasminogen activator (t-PA)(-1.71); A rat novel protein which is expressed with nerve injury (-1.51)

Table 3.6: Real-time quantitative PCR confirmation on 7 genes selected from microarray data (Appendix 22).

	Genbank accession number	Axotomy + vehicle (fold change, compared with Unoperated)		Axotomy + NT-3 (fold change, compared with Axotomy + vehicle)	
		Microarray	Real-time PCR	Microarray	Real-time PCR
Neurofilament-L	M25638	-2.12	-2.17	1.15	-1.14
Neuropeptide Y	M15880	54.53	180.68	-1.02	-1.08
5HT3 receptor	U01227	-1.85	-2.56	1.03	1.06
$p75^{NTR}$	X05137	-1.69	-3.02	1.32	1.31
Heat shock protein 70	Z75029	1.05	1.31	-1.14	-1.12
Peripheral-type benzodiazepine receptor	J05122	4.31	3.41	1.06	-1.11
Glutamate receptor subnit 5-2	M83561	-2.13	-1.64	-1.37	-1.39





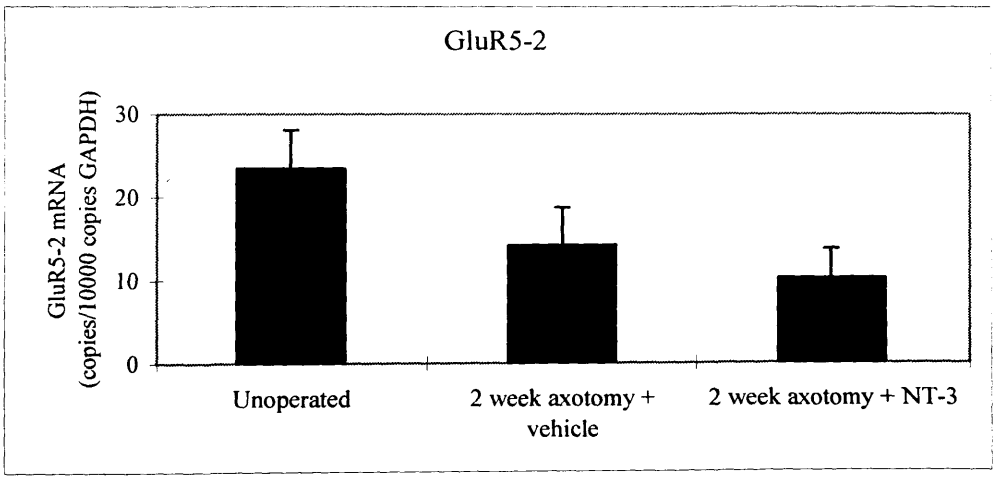


Figure 3.22 (for legend see page 159)

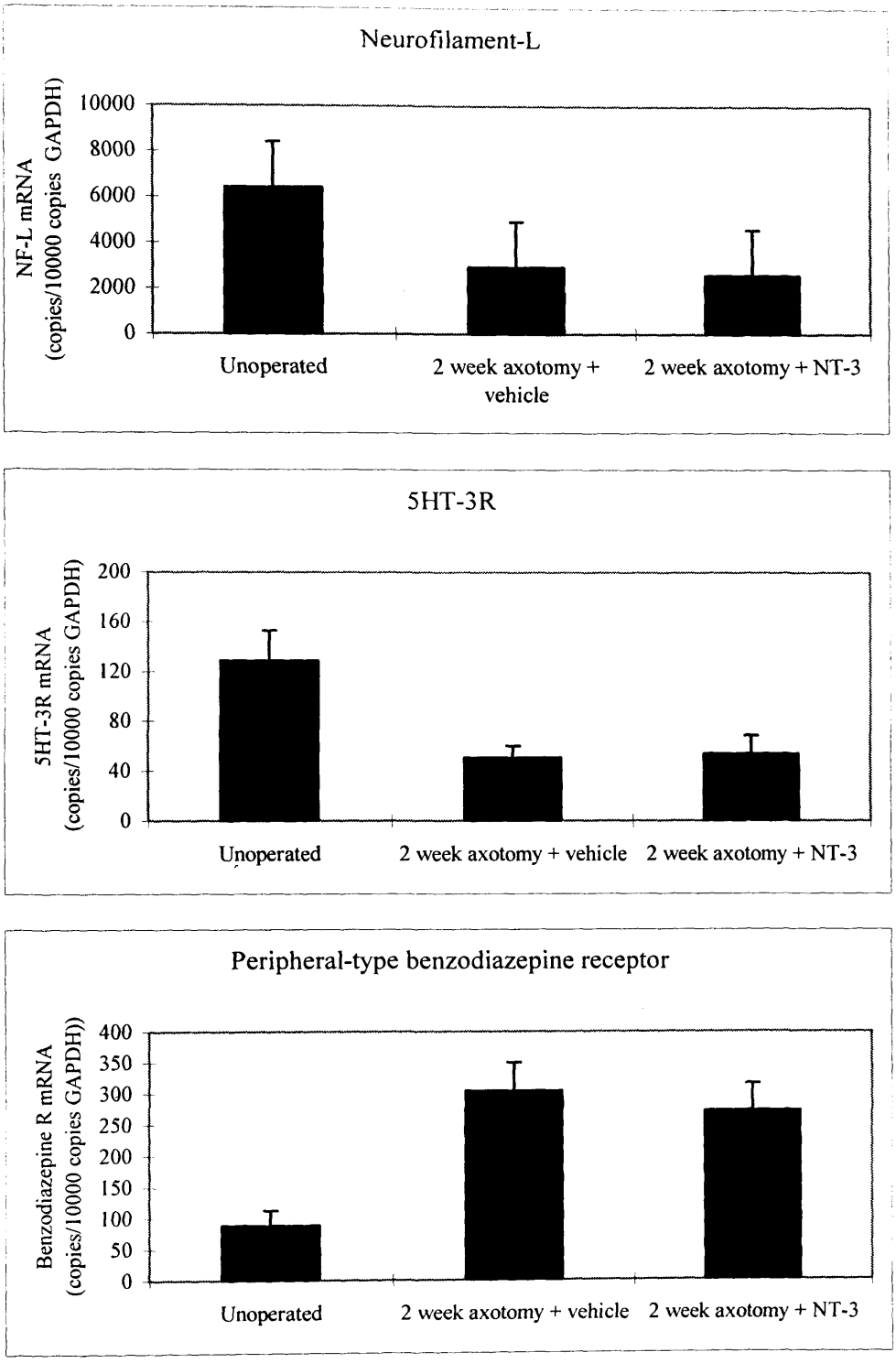


Figure 3.22 (for legend see next page)

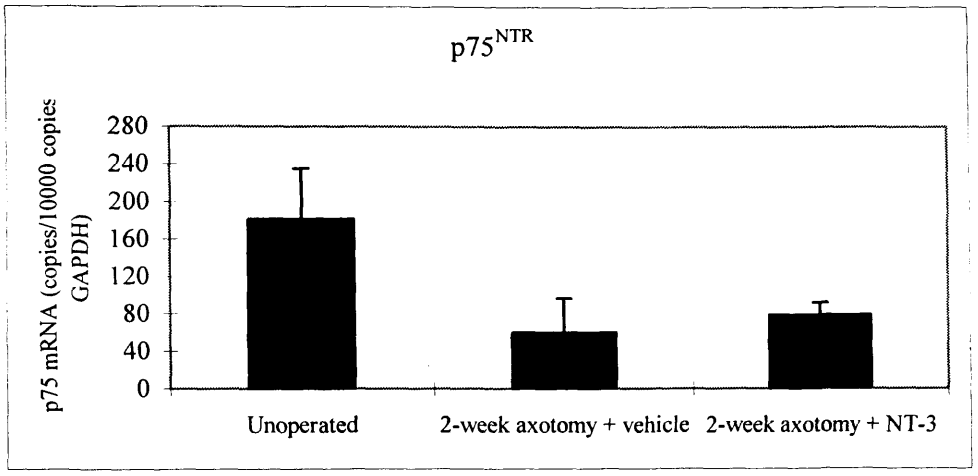


Figure 3.22: Real-time quantitative PCR confirmation of 7 selected genes from microarray data.

CHAPTER 4: DISCUSSION

In this section I will present and discuss the morphological, immunohistochemical and molecular results of my work and provide evidence in support of the hypothesis that NT-3 enhances neuronal regeneration in DRG after sciatic nerve transection. The results will be compared with relevant data from the literature, and the molecular events underlying the neuronal response of axotomised DRG after administration of NT-3 will be discussed.

4.1 Effects of axotomy and systemic NT-3 administration on neuronal number and apoptosis in adult rat dorsal root ganglia

4.1.1 Methodological considerations

Using a stereological method, the physical disector, neuronal loss was established in this study by calculating the ratio between neuronal number in the ipsilateral L4 & L5 and contralateral DRGs. This approach was based on the finding that neither degenerating or apoptotic neurons, nor any indication of neuronophagia, was seen in any section of the serially-sectioned contralateral ganglia. The physical disector technique provided unbiased estimates of absolute DRG neuronal numbers in unoperated animals comparable to those obtained by the optical disector technique (Tandrup, 1993, 1995; McKay Hart et al., 2002). My work was carried out on ganglia sectioned at 4 μm and stained with cresyl fast violet, using a plane of section parallel to the longitudinal axis of the ganglion; this thickness is ideal for distinguishing "tops" of nuclei, which were between 10-18 μm in diameter, in the reference sections. Using nucleus as a counting unit which every cell has exactly one, this method provided accurate estimates of neuronal numbers when it was compared with a

serial reconstruction method based on nucleolar counting (Coggeshall et al., 1994). Stereology uses geometric probabilities and can provide simple, reliable and efficient quantitative methods for assessment of normal quantitative morphology and evaluation of degeneration and cell loss in the DRG. Systemic, uniformly random sampling at all levels is the principle (see Tandrup, 2004). Because the sample of counting fields should represent the whole ganglion cell population and the uneven distribution of DRG neurons, which are clustered between the fiber fundles, more fields are necessary than in situations with more homogeneous distributions. In addition, a microcator is necessay for measurements of section thickness. Because of the variation among ganglia, it is sufficient to count 100 cells to obtain a stable number estimate (Gundersen et al., 1988).

As it was shown that most, if not all, neurons with the morphological features of apoptosis could be stained with TUNEL technique in frozen sections (McKay Hart et al., 2002). Apoptosis is a term originally used to describe a particular morphology related to a special condition of the cell, and the use of morphological criteria of apoptosis is now well established, especially when applied to well-fixed tissue (Groves et al., 1997; Portera-Cailliau et al., 1997; Groves et al., 1999; see Clarke, 1999). In this study, the estimates of neuronal numbers were obtained using stereology with the evaluation of neuronal apoptosis in the same ganglia, using morphological criteria on cresyl fast violet stained sections. Direct observation of apoptotic neurons provides information about the incidence of neuron death at a particular time point. All neurons identified as apoptotic by their morphology also showed activated caspase-3 immunoreactivity in sections immunostained for it, confirming that apoptotic pathways were activated in these cells.

4.1.2 Neuronal number and apoptosis after sciatic nerve transection and systemic NT-3 **administration**

In this study, the ratio of neuron number between right (L4 & L5) and left (L4 & L5) DRGs was used to analyse the neuronal loss after axotomy and the effects of NT-3 treatment. Because I used the rats with the same initial weights for 2-week and 4-week study, there was a variation of age (around 2 weeks) when the animals were sacrificed. As the postnatal neurogenesis in rat DRG has been observed by some authors (Cecchini et al., 1995; Popken and Farel, 1997; Farel, 2002, 2003), it is not appropriate to compare ipsilateral and contralateral DRGs with those in untreated rats. Contralateral cell increase stimulated by NT-3 administration cannot be ruled out, which may lead to the underestimation of neuronal replacement in the axotomised ganglia. However, there was no evidence of neuron gain in contralateral ganglia from BrdU labelling in our previous study (Groves et al., 2003) and nestin immunostaining in this study. The ratios in unoperated rats shows a significantly greater number (about 7%) of neurons on the right side than on the left. Mid-thigh sciatic nerve transection with ligation induced a similar apoptotic rate in both vehicle- and NT-3-treated groups at 2 weeks after axotomy. This resulted in approximately 15-20% neuronal loss in injured L4 & L5 DRGs at 4 weeks in rats not treated with NT-3 (calculated from the right : left ratio in neuronal numbers of around 0.90 at 4 weeks after axotomy and 1.07 in unoperated ganglia); at this time the incidence of apoptosis stayed at a similar level as at 2 weeks in any of the no treatment, vehicle-treated and NT-3-treated groups. An identical lesion has been reported to produce a neuronal loss of around 14% at 8 weeks and 37% at 32 weeks after injury (Tandrup et al., 2000); in that study neuronal loss was estimated by absolute counts in bilateral L5 DRGs which are prone to

have larger variation than using right : left ratio of neuronal numbers in L4 & L5 DRGs as I did in my study.

On the other hand, systemic NT-3 administration for 4 weeks at the dose of 1.25mg/4 weeks and 5mg/4 weeks prevented the decline of the neuronal number in ipsilateral ganglia, although it did not have any effect upon neuronal apoptosis at any of the time points studied. However, the absolute number of neurons lost via apoptosis can only be calculated if the time taken by the process were known. In this respect, only data from *in vitro* studies are available.

Work done on sympathetic neurons showed that the time taken for cells undergoing apoptosis *in vitro*, induced by NGF withdrawal, to become 'ghost cells' and disappear is around 2-3 hours after initiation of the process (Edwards and Tolkovsky, 1994); similar times were found for other neuron populations (see Clarke, 1999). Although the results of *in vitro* study have to be applied with caution, it is possible to hypothesize that if the time of the apoptotic process *in vivo* is similar to that *in vitro*, it would lead, over the relatively long time periods of my observations, to a significant loss of neurons. Because caspase pathway is part of a cascade, it probably does not vary in the time taken to completion once started. Indeed, on the basis of 15 apoptotic neurons seen in the ipsilateral DRGs at any time and of a duration of apoptosis elimination of 3 hours, it could be calculated that 2 weeks after axotomy and vehicle administration, the loss could be of at least 100-150 every 24 hours, resulting in a loss of 1400-2100 neurons between 2 weeks and 4 weeks after axotomy. It is known that axotomy-induced apoptosis begins at around 1 week (Groves et al., 1997) or earlier (McKay Hart et al., 2002), therefore the neuronal loss at 4 weeks after axotomy would be greater than 1400-2100 neurons. In the group treated with 1.25mg NT-3

for 4 weeks, around 19 apoptotic neurons were seen in the ipsilateral DRGs at 2 weeks after axotomy and 11 apoptotic neurons at 4 weeks. Assuming the absolute number of neuronal loss can be calculated on the basis of mean apoptotic neuron number at these two time points, similar conclusions can be drawn as those in the vehicle-treated groups.

The finding that apoptotic neurons in both NT-3- and vehicle-treated groups were immunoreactive for activated caspase-3, which is one of the final steps in the caspase cascade, confirms that caspase-3 was activated throughout the time period when apoptotic neurons were morphologically recognisable. This shows that NT-3 treatment did not change the apoptotic pathway, and so would not have slowed down the process.

The lack of effect of exogenous NT-3 upon neuronal apoptosis following axotomy raises the question of how it prevents the decline in neuronal number in the ipsilateral ganglia. The identical incidence of apoptosis at 2 weeks in NT-3- and vehicle-treated groups indicates that NT-3 treatment did not delay the onset of neuronal apoptosis, which normally starts around 1 week after axotomy (Groves et al., 1997; McKay Hart et al., 2002). It can be concluded that NT-3 does not share the effects of glial-derived neurotrophic factor (GDNF), which alone was shown to reduce the number of neurons undergoing apoptosis in cultured adult DRG explants whilst NGF, BDNF and NT-3 did not (Edstrom et al., 1996; Leclere et al., 1997); these results suggest that for most DRG neurons, signalling through the neurotrophin receptors does not influence neuronal apoptosis *in vitro*. My results show that NT-3 does not influence neuronal apoptosis *in vivo*.

As apoptosis persists in NT-3-treated rats at the same rate as in untreated, the replacement of dying DRG neurons either by newly-formed or newly differentiated neurons must be considered to explain this. Neurogenesis in postnatal DRG has been

studied by a few researchers; Farel (2002, 2003) reported that the number of rat lumbar DRG neurons increased by 20% between postnatal day (P) 11 and P100, and by 35% between P1 and P100 using counts and 3 dimensional counting. Devor et al. (1985) also showed a 50% increase of DRG neurons between 85 and 490 postnatal days using corrected profile counts. BrdU, which can incorporate into DNA during S phase in dividing cells as a mitotic marker, was used in some studies to observe proliferating precursor cells (Ciaroni et al., 2000; Farel, 2003; Groves et al., 2003). BrdU has been shown to be specific for neurons that are derived from dividing cells, rather than neurons undergoing DNA repair, at the dose used (Cooper-Kuhn and Kuhn, 2002). Ciaroni et al. (2000) found some BrdU-positive rounded or bell-shaped cells in L4 DRGs of adult vitamin E-deficient rats between 4 and 5 months of age; some of these cells showed neuronal phenotype and were suggested to be transitional neuroblasts. However, these cells were scarce and were never found to have morphological features of well-differentiated neurons. Others have reported an unchanged DRG neuronal number in relation to age (Chimelli and Scaravilli, 1986; Pover et al., 1994; Mohammed and Santer, 2001); an earlier study using [^{3}H] thymidine to label dividing cells showed that neurogenesis in DRG is complete by embryonic day 15 (Lawson et al., 1974). St. Wecker et al. (1994) reported that the neuronal numbers doubled during postnatal life in frog, whereas no neurons were labelled with [^{3}H] thymidine, a method which only identifies cells produced from cell division during the period of administration. Moreover, the increase of neuronal number in DRG observed by Farel (2002) was also suggested to be a result of late differentiation because subcutaneously-injected BrdU did not label any DRG neurons between P1 and P15 when new neurons were expected to be added at the greatest rate (Farel, 2003).

In addition to the possible neurogenesis in normal DRG, we (Groves et al., 2003) have recently reported more than 15-20% neuronal loss in DRG, using physical dissector technique to count neurons, at 1 month and 2 months after sciatic nerve crush, but no significant loss observed at 3 months, suggesting that replacement of lost neurons occurs in injured ganglia without any treatment. The small number (0.04%) of neurons in axotomised L4 and L5 DRGs labelled with BrdU suggested either that cell proliferation was insufficient to explain the neuronal replacement or that the BrdU technique is unsuitable for identifying new neurons. Moreover, Ljungberg et al. (1999) found that intrathecal administration of NT-3 or NGF following transection of an intercostal nerve resulted in a 25% increase in the number of DRG neurons that transported a tracer from the injury site. These data suggests that neuronal addition may be further stimulated by neurotrophins in injured ganglia.

In PNS, neuronal precursors may derive from self-renewing, multipotent neural crest stem cells (NCSCs) in the embryo, which then maintain a limited ability to proliferate and differentiate into neurons. Indeed these precursors have been extracted from adult mouse DRG and found to be capable of differentiating into neurons *in vitro* when exposed to neurotrophic factors including NGF and NT-3 (Namaka et al., 2001). During embryonic development of mouse DRG, NT-3 is thought to trigger the final differentiation of sensory neuroblasts into post-mitotic neurons (ElShamy et al., 1998). This can be promoted through a truncated isoform of trkC that lacks the catalytic kinase domain (known as trkC NC2; (Menn et al., 1998)), in conjunction with $p75^{NTR}$ (Hapner et al., 1998). It is possible that NT-3 has similar actions *in vivo*, under certain conditions, on any sensory neuron precursor cells present in adult DRG, such as following peripheral nerve injury. All these data

indicate that stem cells and precursor cell/undifferentiated neuron type may exist in adult rat DRG without undergoing cell division to any significant extent (Farel, 2002, 2003), but may undertake the pathway towards final maturation/ differentiation when stimulated by certain factors, including peripheral nerve injury alone or, to higher extent, following administration of neurotrophic factors.

NGF and NT-3 have previously been shown to prevent the decline in DRG neuronal number after peripheral axotomy when administered intrathecally or to the proximal stump (Otto et al., 1987; Rich et al., 1989; Groves et al., 1999; Ljungberg et al., 1999). Among these studies, Otto et al., Rich et al., and Ljungberg et al. estimated neuron numbers by corrected numbers of neurons from counts of nucleolar profiles; Groves et al. used stereological methods. It has been suggested that this effect is mediated through their high affinity receptors, trkA and trkC respectively, whose activation has been shown to be neuroprotective and prevent neurons from undergoing injury-induced apoptosis after axotomy, at least in neonates (Ernfors et al., 1994; Smeyne et al., 1994). In contrast, Tandrup et al. (1999), using stereological technique, reported that systemic NGF treatment by subcutaneous injection for 1 month had no apparent effect on neuronal loss in L5 DRGs after spinal nerve transection. Different counting methods may explain the different results obtained by systemic and intrathecal NGF application. Systemic NT-3 treatment in my study appears to stimulate neuronal replacement instead of affording neuroprotection as shown by the unchanged incidence of neuronal apoptosis. It is possible that this may be due to different intracellular signalling pathways being activated by axonal and perikaryal trkC receptors (Watson et al., 2001)(see 1.2.12), both of which (Erk1/2 and Erk5 pathways) were assumed to be activated concurrently by systemic NT-3 administration rather than the

activation of only one of them by other methods of application such as intrathecal infusion and administration to the proximal stump.

4.1.3 Effect of systemic NT-3 administration on nestin mRNA expression and immunoreactivity

Using real-time PCR quantitation and immunohistochemistry for nestin, this study showed increased nestin expression in some small neuron-like oval-shaped cells (mean diameter: 20-21μm) in vehicle-treated injured DRG; there were no neurons expressing nestin protein in the contralateral ganglia or ganglia from unoperated rats. Systemic NT-3 treatment for 4 weeks significantly up-regulated mRNA expression of nestin and increased the incidence of nestin-immunoreactive neurons in ipsilateral ganglia. All of these nestin-expressing cells are also immunoreactive for a neuronal marker, β-III tubulin, and some of them express trkA, trkC, or CGRP. Nestin immunoreactivity was seen in a large number of satellite cells surrounding large neurons in unoperated animals, and no change was observed in satellite cell expression of nestin after axotomy or NT-3 treatment.

Nestin is a class of VI intermediate filament protein mainly expressed in proliferating progenitor cells, neuron-restricted precursor cells, glial-restricted precursors in mammalian and rodent developing CNS and PNS as well as developing muscle and myocardial cells (Hockfield and McKay, 1985; Lendahl et al., 1990; Sjoberg et al., 1994; Dahlstrand et al., 1995). When these precursor cells differentiate into neuronal and glial cells, nestin is down-regulated and replaced by cell type-specific intermediate filament, such as neurofilaments or GFAP (Frederiksen and McKay, 1988). In the adult brain, nestin can be found in cells in restricted areas which exhibit postnatal neuro- or gliogenesis such

as the hippocampus, subventricular cells and olfactory neuronal progenitor cells (Morshead et al., 1994; Doyle et al., 2001). Nestin has also been detected in bone marrow stromal cells (BMSC) and in various pathological conditions including CNS tumours, excitotoxic, ischaemic and traumatic cerebral injury (Dahlstrand et al., 1992; Li and Chopp, 1999; Sanchez-Ramos et al., 2000).

In my study, nestin-immunoreactive cells had a mean diameter of around 20-21 μm, much bigger than satellite cells. Co-expression of nestin with β-III tubulin, which is considered to be one of the earliest neuron-associated cytoskeletal marker proteins (see Katsetos et al., 2003), in these cells can be interpreted as evidence for neuronal replacement after peripheral nerve transection. The expression of trkA or trkC in some nestin-immunoreactive cells implies that neurotrophins may play a role in these cells, or that these cells are responsive to exogenous or endogenous neurotrophins. CGRP is a neuropeptide that is synthesised in approximately 50% of the small- and medium-size DRG neurons, especially trkA-expressing neurons (Averill et al., 1995), and is transported along the axon to the periphery and dorsal horn to modulate nociception and inflammation (Ju et al., 1987; McNeill et al., 1988; Heppelmann and Pawlak, 1997). The appearance of nestin- and CGRP-expressing cells suggests that intraganglionic sprouting of CGRP axons may originate from these neurons and may be implicated in the development of chonic neuropathic pain (Hu and McLachlan, 2000).

Because nestin immunoreactivity was not observed in axons in this study, it is not known whether nestin-immunoreactive neurons extend axons beyond their immediate vicinity to the spinal cord or to the periphery. Unless a specific marker of newly formed

neurons that is present in axons is discovered, it is very difficult to investigate the sprouting of axons from nestin-immunoreactive cells after injury.

4.1.4 Possible source of neuronal replacement

As mentioned in 4.1.2, one possible source of the neuronal replacement observed in the present study is the late maturation of immature nerve cells. The lack of difference between animals treated with 1.25 and 5mg could reflect the fact that the lower dose of NT-3 applied in this study (1.25mg/4 weeks) may be maximal and sufficient to trigger all the possible neuronal precursors to leave the cell cycle and differentiate. A more intriguing possibility is that the neuronal replacement is related to the number of apoptotic neurons.

The proliferation and activation of satellite cells after nerve injury, as reviewed in 1.3.5, could also imply their involvement in neuronal replacement after axotomy and NT-3 treatment. The injury-induced synthesis of neurotrophic factors, such as NGF and NT-3, in both neurons and satellite cells (Zhou et al., 1999b), and the expression of trkA and trkC I observed in satellite cells of axotomised ganglia suggest that there may be increased communication between neurons and their surrounding satellite cells and satellite cells may respond to NT-3 treatment.

Bone marrow is another potential source of neuronal precursors capable of moving into adult mouse DRG and differentiating into neurons. Sanchez-Ramos (2002) reviewed the literature in which cells from postnatal bone marrow were induced to proliferate and differentiate into glial cells and neurons in CNS. Various agents were successfully used to induce neural differentiation *in vitro*, including neurotrophins (NGF, BDNF and NT-3),

retinoic acid and other neurotrophic factors (Sanchez-Ramos et al., 2000; Reyes and Verfaillie, 2001; Koyama et al., 2003). *In vivo* studies in which adult bone marrow cells were transplanted into systemic circulation also showed that the administered bone marrow cells could migrate to normal brain and differentiate into neuron-specific antigen-expressing cells (Brazelton et al., 2000; Mezey et al., 2000). In rodents, intracerebral grafting and intravenous infusion of bone marrow stromal cells (BMSC) might be used to facilitate the recovery of neurologic deficits of stroke, CNS trauma and Parkinson Disease (Li et al., 2000b; Chen et al., 2001); however, whether the therapeutic benefit comes directly from newly-formed neurons with functional activities or indirectly from the trophic factors and cytokines elaborated from some of the transplanted cells is unclear. Human haematopoietic bone marrow cells have been reported to express trk receptors including the catalytic and truncated isoforms of trkC (Labouyrie et al., 1999), suggesting that NT-3 may exert effects on bone marrow cells through trk signalling. Corti et al. (2002) demonstrated that numerous transplanted bone marrow cells were seen in adult normal DRGs (around 20 cells per coronal section of ganglia) at 3 months after transplantation. A small number of them were found to express neuronal marker acquired phenotypes, including class III β-tubulin, neural-specific nuclear antigen (Neu-N) and neurofilament (NF); these neuronal cells were small, round and with short processes (Corti et al., 2002). These results showed that bone marrow cells could acquire neuronal phenotypes in uninjured DRG as previously shown in brain (Brazelton et al., 2000; Mezey et al., 2000). Taken together, bone marrow could be a potential source of neuronal replacement in axotomised DRG in my study, and NT-3 may be an enhancing factor.

The functional aspects of DRG neuronal replacement are unknown. Because nestin immunoreactivity was not observed in axons in this study, it is not known whether nestin-immunoreactive neurons extend axons beyond their immediate vicinity to the spinal cord or to the periphery. Unless a specific marker of newly formed neurons that is present in axons is discovered, it is very difficult to investigate the sprouting of axons from nestin-immunoreactive cells after injury. However, retrograde tracing and confocal microscopy may be helpful in investigating whether nestin immunoreactive neurons extend axons and where these axons project to. If there is any axonal sprouting from nestin-immunoreactive neurons within DRG, they may be relevant to the development of neuropathic pain associated with nerve injury (see Ramer et al. 1999).

4.2 Effects of axotomy and systemic NT-3 administration on neuron size and gene expression of neurofilaments

4.2.1 Methodological considerations

Profiles of around 600 neurons were measured in each ganglion examined. Cell sizes in wax-embedded ganglia are reduced compared with those in frozen sections (McCarthy and Lawson, 1990), but the overall profile of sizes is unaffected (Lawson and Harper, 1985). Because of the bimodal distribution of neuron size, I used a non-parametric test to compare the size profiles of neurons between ipsilateral L4 & L5 DRGs and contralateral ganglia. A previous study has shown that axotomy has no detectable contralateral effect on the neuronal volume from 4 days to 45 days after spinal nerve transection (Vestergaard et al., 1997).

The general decrease in neuron size may mask selective increases or decreases among particular size categories. To examine this possibility, Farel. (2002) presented the disproportionate increase in small neurons in L4 DRGs between postnatal day 1 (P1) and postnatal day 100 (P100) rats by normalising his data by the mean and analysing the absolute number of neurons below the mean. I used the same method to see whether axotomy and NT-3 administration produce effects on the proportion of a specific subpopulation of neurons.

4.2.2 Neuron size profile and neurofilament mRNA in axotomised DRG

It has been reported that rat DRG cells continue to increase in diameter in proportion to the increase in body surface area for at least 100 days after birth (Donaldson and Nagasaka, 1918; Lawson et al., 1974). In addition to the general increase of neuronal size, Farel (2002) noted increased number of neurons in L4-6 DRG between P1 and P100 and the added neurons fell disproportionately into the population below that of the mean size within the ganglion.

My results of neuron size distribution showed a shrinkage of neurons of all sizes in the ipsilateral ganglia since 2 weeks after axotomy. Previous reports showed a 15-30% decrease in cell volume in neurons of all sizes from one to 3 weeks after the same procedure, and a 33% decrease 4 days after spinal nerve transection (Rich et al., 1987; Vestergaard et al., 1997; Bergman et al., 1999). The neuron size distribution was shown to shift to the left 2 weeks and 4 weeks after sciatic nerve transection.

Moreover, Vestergaard et al. (1997) not only reported shrinkage of DRG neurons at 4, 8 and 15 days after spinal nerve axotomy, but also observed a partial restoration of mean

volume at 45 days; the disappearance of some small neurons was one possible explanation. Rich et al. (1989) found that neuronal death was greatest among smaller neurons with diameters between 16 and 28 μm following sciatic nerve transection.

Concomitantly, the mRNA expression of neurofilament-light chain (NF-L) examined by microarray analysis and real-time PCR confirmation also revealed a more than 2 fold decrease at 2 weeks after axotomy. The expression of mRNA for neurofilament, which is a major determinant of the size of the neuronal perikaryon and axon (Friede and Samorajski, 1970; Portier et al., 1983; Hoffman et al., 1987), has already been reported to be down-regulated in DRG neurons after transection of either central or peripheral branch axons of DRG neurons (Greenberg and Lasek, 1988; Wong and Oblinger, 1990a). Since NF-L is a neuron-specific protein, axotomy-induced alterations in the levels of the mRNAs encoding this protein reflect corresponding changes in its expression by DRG neurons.

4.2.3 Effects of systemic NT-3 administration on neuron size and neurofilament mRNA

It has been shown that NGF from target organs plays an important role in determining neuronal size; NGF administration for 3 months to the proximal stump of the transected sciatic nerve significantly decreased the degree of neuronal atrophy from 28% to 13% (Rich et al., 1987). Whether any of the other neurotrophins, including NT-3, could rescue the axotomy-induced neuronal atrophy was not clear. In my study, systemic NT-3 administration at either dosage did not produce any significant effect on the shrinkage of neurons, and the NT-3 treatment for 2 weeks did not significantly regulate the transcripts of NF-L and NF-H, which were examined by microarray. Therefore, I suggest that systemic NT-3 administration does not significantly rescue the axotomy-induce neuronal atrophy, at

morphological or molecular levels. Although NT-3 may interact with trkA in addition to its preferred receptor, trkC, it still cannot replace the rescue effect that NGF has on neuronal atrophy.

4.3 Effects of systemic NT-3 administration on the mRNA expression of neurotrophin and neurotrophin receptors

The results discussed in section 4.1 and 4.2 show that NT-3 may stimulate neuronal replacement in DRG after sciatic nerve transection; however they do not provide information about the molecular mechanisms activated by this neurotrophin. In particular, it is not clear whether NT-3 acts through its high-affinity receptor, trkC, and low-affinity receptor, $p75^{NTR}$ and the extent of the contribution by other neurotrophins. This section of the discussion will analyse my data on the expression of three main neurotrophins, NGF, BDNF and NT-3, and their receptors after axotomy and systemic NT-3 administration.

4.3.1 Methodological considerations on real-time PCR

Compared with conventional PCR, real-time quantitative PCR has some advantages: (1) increased speed due to the reduced cycle times, removal of separate post-amplification manipulation and detection procedures; (2) increased sensitivity because of the use of sensitive fluorescence (SYBR in this study) detection equipment; (3) good reproducibility.

Successful quantitative RT-PCR is mainly dependent on the quality of RNA. In this study, RNA was extracted from whole ganglia which contained different cell types including sensory neurons, satellite cells, Schwann cells, mast cells, fibroblasts, perineurial and endothelial cells. RNA samples were purified using Qiagen RNeasy columns, which

are supposed to isolate pure RNA but were shown to have DNA contamination to some degree (Bustin, 2002). For preventing the inaccurate quantification resulting from DNA contamination, DNase was routinely applied during RNA extraction in this study and removed during the subsequent wash steps. In order to decrease the variations due to mispriming and primer dimerisation, thermostable polymerases, requiring heat activation at 95 °C before the first cycle of RNA, were used.

Using any quantitative RT-PCR method inevitably results in averaging the expression of all cell types and masking the minor change of the expression profile of a specific cell type. Laser capture microdissection (LCM) should be introduced to select individual cells by directing a brief laser pulse at desired cells within a formalin-fixed paraffin-embedded tissue section on a glass slide, followed by RNA extraction and quantification analysis. However, even this method could not avoid contamination by satellite cell cytoplasm. I overcame this limitation by adding to real-time quantitative PCR *in situ* hybridisation to identify the cell type responsible.

Data normalisation is an important process to make samples from different experimental animals comparable. GAPDH was shown to be expressed at a constant level in DRG after sciatic nerve injury (Gallinat et al., 1998; Araki et al., 2001; Macdonald et al., 2001), and my data from microarray study also demonstrated that GAPDH did not change its mRNA expression in injured state, validating the use of GAPDH as a housekeeping gene in real-time PCR data analysis.

4.3.2 mRNA expression of neurotrophins after axotomy

In my study, the mRNAs for NGF, BDNF and NT-3 were detected by real-time PCR and *in situ* hybridisation in DRG neurons, but not in satellite cells, from unoperated animals, as reported by others using *in situ* hybridisation, RT-PCR and northern blot analysis (Sebert and Shooter, 1993; Zhou et al., 1999b). However, Shen et al. (1999) failed to detect NT-3 mRNA in normal or axotomised DRG by ribonuclease protection assay. In other earlier literatures using *in situ* hybridisation, RT-PCR and northern blot analysis, neither NGF nor NT-3 mRNA was shown to be present in a detectable amount in normal DRG (Davis et al., 1987; Ernfors et al., 1990; Wetmore and Olson, 1995). Different methodologies which have different sensitivities may explain the different results.

Concomitantly with the expression of the high-affinity receptors, trkC and trkA, in specific subpopulations of DRG neurons, NT-3 appears to have a greater effect on myelinated sensory axon regeneration than NGF, whereas NGF seems to stimulate unmyelinated axon regeneration (Bradbury et al., 1999; Romero et al., 2001). As expression of mRNA for trkA and trkC was occasionally seen in satellite cells in unoperated DRG, it could be proposed that these locally-synthesised neurotrophins may be related to trophic support to normal DRG neurons and satellite cells.

My data show that the expression of mRNA for NGF increased at 1 day and 1 week after axotomy in vehicle-treated ipsilateral ganglia, which was consistent with previous results (Sebert and Shooter, 1993; Zhou et al., 1999b). The up-regulation of NGF in axotomised DRG, was reported to be largely due to increased synthesis in satellite cells, which did not express detectable levels in uninjured ganglia (Zhou et al., 1999b). However, using *in situ* hybridisation, I found increased expression of NT-3 and NGF in small-to-medium neurons in addition to some satellite cells. The injury-induced increase in mRNA

expression of neurotrophins implies the importance of local neurotrophin synthesis within DRG, which may provide autocrine and paracrine effects, instead of target-derived trophic support from the periphery in injured DRG.

Lee et al. (1998) reported that the NGF protein level was decreased at 6 hours after tight ligation of spinal nerves, but recovered to almost normal levels at 2 days, suggesting that the increased synthesis of NGF within the injured DRG contributes to the recovery; another potential source of endogenous NGF synthesis is the proximal stump of transected sciatic nerve (Heumann et al., 1987).

My data show that BDNF mRNA expression increases in neurons of various sizes at 1 day after axotomy, but not in satellite cells, which is in keeping with other researchers' results (Michael et al., 1999; Karchewski et al., 2002). This increase lasted for less than one week. In uninjured ganglia, BDNF is expressed in a number of trkA-expressing nociceptive sensory neurons and is transported anterogradely to the dorsal horn of the spinal cord where it is located in C-fibre terminals (Michael et al., 1999). The increase in BDNF mRNA and protein in trkB- and trkC- expressing neurons in axotomised ganglia and the enhanced anterograde transport of BDNF to the spinal cord following sciatic nerve injury may contribute to increased synaptic transmission and modulates somatosensory pathways (Tonra et al., 1998; Michael et al., 1999; Thompson et al., 1999). In contrast to the increased levels of BDNF mRNA, the mRNA expression of its preferred receptor, trkB, was down-regulated 2 weeks after axotomy. This suggests that the increased synthesis of BDNF in injured ganglia may play a more important role in spinal cord than in the axotomised DRG.

In my study, mRNA levels of NT-3 increased only at 1 day after axotomy; *in situ* hybridisation localised its expression mainly in small and some large neurons as well as satellite cells. Because of its relatively low expression in normal DRG, only Zhou et al. (1995, 1999b), using RT-PCR, *in situ* hybridisation and immunohistochemistry, reported a detectable level of NT-3 mRNA and protein in normal small and large DRG neurons and an up-regulation of NT-3 synthesis mostly in satellite cells at 2 days after sciatic nerve transection, which lasted for 2 months. The satellite cell-derived NGF and NT-3 may contribute to the trophic support for regenerating neurons as well as those trkA- and trkC-expressing satellite cells. Compared to the down-regulation of trkA and trkB mRNA following axotomy, the up-regulation of trkC mRNA in DRG neurons of all sizes for 2 weeks and its expression in satellite cells in uninjured ganglia suggests the importance of trkC signalling in axotomised ganglia.

The axotomy-induced synthesis of NGF and NT-3 in DRG has also been implicated in the formation of sympathetic nerve sproutings in the axotomised DRG from 2 weeks after injury (Zhou et al., 1999b; Deng et al., 2000). These grow around large diameter neurons, and have been linked to the development and maintenance of neuropathic pain after nerve injury (Frostick et al., 1998; Zhou et al., 1999b). Sympathetic neurons express both trkA and trkC, and require NGF and NT-3 for neuronal survival and differentiation during their development (Farinas et al., 1994; Zhou and Rush, 1995). Zhou et al. (2000) showed systemic administration of NGF and NT-3 antisera prevented sympathetic nerve sprouting and attenuated allodynia as assessed by foot withdrawal responses evoked by von Frey hairs (Zhou et al., 2000).

4.3.3 mRNA expression of neurotrophin receptors after axotomy

My finding that axotomy decreases the mRNA expression of trkA is in keeping with reports by previous authors using Northern blotting, RNase protection assays, *in situ* hybridisation, microarray and immunohistochemistry after sciatic nerve or spinal nerve transection (Krekoski et al., 1996; Bergman et al., 1999; Shen et al., 1999a; Xiao et al., 2002); however, some studies in which only crush injury was applied found no change in trkA mRNA expression (Ernfors et al., 1993; Sebert and Shooter, 1993). In normal DRG, trkA-expressing sensory neurons are mainly small-sized (McMahon et al., 1994). Shen et al. (1999) reported that the increased amount of NGF protein synthesis did not correlate well with the recovery of trkA mRNA expression in the DRG after axotomy (Lee et al., 1998; Shen et al., 1999b), suggesting that NGF signal transduction through the soma membrane is different from that taking place through axon terminals at the periphery in intact state, or that some other factors in addition to NGF may also regulate trkA mRNA levels. It has recently been reported that different mitogen-activated protein kinase (MAPK) signalling pathways are activated by NGF during retrograde signalling and direct stimulation of the cell soma (Watson et al., 2001). An *in vivo* study showed that intrathecal NGF administration partly rescued the axotomy-induced decrease of trkA mRNA (Verge et al., 1992). The transient elevation of BDNF and NT-3 mRNA expression in axotomised DRG may not contribute much to the late recovery of trkA levels.

In this study, trkB mRNA was down-regulated at 2 weeks after axotomy and returned to control levels by 4 weeks, which is consistent with the study by Bergman et al. (1999) using *in situ* hybridisation and immunohistochemistry. Using Northern blotting, others have reported increased trkB mRNA 1 week after crushing the sciatic nerve (Ernfors

et al., 1993). The difference could derive from variations in the sciatic nerve lesions used and the quantitative methods. The primers for PCR and oligonucleotide probes for *in situ* hybridisation were designed from sequences of catalytic isoforms of trkB and trkC (see 2.3.5), which have tyrosine kinase domains and display tyrosine phorphorylation in response to BDNF and NT-3 respectively. The mechanism of trkB regulation is not known, although local neuronal synthesis of BDNF is known to be the main source of this neurotrophin. This is in contrast to the synthesis in target organs of NGF and NT-3; this may explain why the axotomy did not initiate the change of trkB mRNA at earlier time points, as did trkA and trkC.

My results found a large increase in ipsilateral trkC mRNA expression in neurons of all sizes, as well as satellite cells, within 24 hours of axotomy. This increase was sustained for at least 2 weeks, which is consistent with the findings by Ernfors et al. (1993), but at variance with another study which used *in situ* hybridisation with probes for all isoforms of trkC and found decreased trkC expression in neurons at 7 days after mid-thigh sciatic nerve transection (Bergman et al., 1999). Different methods of mRNA detection and different targets of examination (catalytic or non-catalytic isoforms) may explain the contradictory results. In normal DRG, my study and those of others showed that trkC was expressed in medium-to-large neurons; the changed pattern of its mRNA expression in axotomised ganglia would imply that trkC signalling may have effects on different subsets of sensory neurons with passing time after peripheral injury.

I found that satellite cells expressed trkA or trkC in both unoperated and injured ganglia; trkA receptor expression has recently been reported in satellite cells of normal DRG (Pannese et al., 2003), but trkC has not. The function of perineuronal satellite cells in

both normal or axotomised DRG is not well studied, and it has been proposed that satellite cells could regulate ion concentration in the microenvironment of DRG neurons (see Pannese, 1994). A recent study discovered the formation of new gap junctions between satellite cells in injured ganglia (Pannese et al., 2003), showing that responses of satellite cells could be triggered by unknown mechanisms after axotomy. The expression of trkA and trkC in satellite cells implies the existence of neuron-satellite cell interactions in intact and axotomised ganglia. The expression of these neurotrophin receptors in some satellite cells also suggests that they have partly neuronal phenotype and may be able to differentiate into neurons.

In the current study, the expression of $p75^{NTR}$ mRNA was decreased in neurons and up-regulated in satellite cells surrounding large diameter neurons in ipsilateral ganglia after sciatic nerve transection, resulting in a marked decrease of $p75^{NTR}$ mRNA in the whole ganglia from 1 day to 1 week after axotomy, as reported by other researchers after the same injury (Krekoski et al., 1996; Zhou et al., 1996; Bergman et al., 1999). $p75^{NTR}$, the prototypic member of the tumour necrosis factor receptor family, is known to be capable of both signalling independently and modifying the binding and signalling capabilities of trk receptors (Hempstead, 2002). In normal lumbar DRG, the $p75^{NTR}$ receptor is co-expressed with most trkA- and trkB-expressing DRG neurons, as well as with half of the trkC-expressing neurons (Wright and Snider, 1995), where it may enhance the binding affinity and neurotrophin selectivity of trk receptors and facilitate the retrograde transport of neurotrophins (Hempstead et al., 1991; Benedetti et al., 1993). The regulating signals are not known, but intrathecal NGF administration was reported to exert a role by partially preventing the decrease of p75 mRNA in axotomised ganglia and increasing the $p75^{NTR}$

transcripts in normal ganglia (Verge et al., 1992). In my study, the up-regulation of neurotrophin mRNA in DRG neurons and satellite cells, particularly NGF mRNA, whose increased expression lasted more than one week, may contribute to the recovery of p75 mRNA seen at 2 weeks after transection. Moreover, down-regulation of $p75^{NTR}$ mRNA expression in injured ganglia after peripheral axotomy may result in a partial loss of specificity for the ligands for trkA and trkB (Bibel et al., 1999; Mischel et al., 2001). This suggests that the responsiveness of trkA- and trkB-expressing neurons to NT-3 may increase in axotomised DRG.

The altered expression of all trk receptors was observed to return to control levels over time after axotomy. Complete recovery of neurotrophin levels by increased local synthesis in DRG may contribute to this on the basis that the elevated endogenous NGF synthesis in the axotomised DRG can fully replace in quantity the decrease of retrograde-transported NGF (Lee et al., 1998). As well as being a result of a deprivation of neurotrophins from target organs, the injury-induced change in trk receptor and $p75^{NTR}$ mRNA could be triggered by other signals being mediated either by neuronal activity or by retrograde transport (Sebert and Shooter, 1993).

4.3.4 Contralateral effects

I found an increased expression of BDNF mRNA and trkA mRNA at 1 day and trkC mRNA at 1 day and 4 weeks, and decreased expression of $p75^{NTR}$ mRNA at 1 day and 1 week in the contralateral DRGs following sciatic nerve transection. Only a limited number of studies on neurotrophins and their receptors have examined contralateral ganglia; they show increased level of NGF mRNA in contralateral DRG at 1 and 4 days after sciatic

nerve crush and decreased trkA-immunoreactive neurons in uninjured DRG at 1 week after spinal nerve transection (Wells et al., 1994; Li et al., 2000a). Unilateral sciatic nerve transection has been shown to produce changes in the contralateral nerve, affecting growth associated protein-43 (Booth and Brown, 1993), structural proteins (neurofilament-L)(Wong and Oblinger, 1990a), and others (e.g. interleukin-1β and transforming growth factor-β1) (Ryoke et al., 2000). It is unclear whether these adaptive reactions in contralateral uninjured DRG serve any functional purpose, but they imply the presence of as yet unidentified communication between the two sides of the body (see Koltzenburg et al., 1999). Possible mechanisms include the delivery of neurotrophic cytokines or stress-related hormones through the general circulation, which has been demonstrated by the increase in NGF mRNA in contralateral cervical ganglia at 3 days after sciatic nerve crush (Fitzgerald, 1983; see Koltzenburg et al., 1999; see Lowrie, 1999). Local bilateral synaptic communication between bilateral motor neurons has been suggested by an experiment which showed that 4 days after injections of the Bartha strain of pseudorabies virus into the medial gastrocnemius muscle of a normal rat or in rats with unilateral L3-L6 dorsal rhizotomies, transneuronally labelled neurons were seen in the ipsilateral L5 spinal laminae I and II and bilaterally in spinal laminae IV-VIII, and X (Rotto-Percelay et al., 1992). Whether local synaptic communication between bilateral sensory neurons exists is not understood.

4.3.5 Effect of systemic NT-3 administration on mRNA expression of neurotrophins

Local and intrathecal administration of NT-3 has been proven to modulate the expression of neuropeptide Y and pituitary adenylate cyclase-activating polypeptide

(PACAP) in DRG neurons after sciatic nerve and spinal nerve injury (Sterne et al., 1998; Jongsma Wallin et al., 2001); however, there are no reports addressing whether systemic NT-3 administration would regulate mRNA expression of neurotrophins, including NT-3 itself, and neurotrophin receptors in axotomised DRG.

Using real-time quantitative PCR, my results showed a temporary increase of mRNA expression of NGF, BDNF and NT-3 in vehicle-treated DRG after mid-thigh sciatic nerve transection as discussed in 4.3.1 and 4.3.2; systemic NT-3 treatment at the dose of 5mg/4 weeks prolonged the up-regulation of these neurotrophins but not of NT-3 itself. *In situ* hybridisation showed the increased expression of NGF to be localised predominantly in small- and medium-sized neuron and some satellite cells around large neurons, and that BDNF was expressed in neurons of all sizes in ipsilateral ganglia following axotomy and NT-3 administration.

Whether NT-3 can regulate the expression of NGF in DRG cells *in vivo* has never been studied. A previous study showed that a single subcutaneous injection of NT-3 at dose of 1mg/kg had no effect on BDNF expression in intact DRG using ribonuclease protection assay (Apfel et al., 1996). My finding that NT-3 administration prolonged the ipsilateral up-regulation in BDNF mRNA appears to be at variance with work by Karchewski et al. (2002) who showed instead that it produced a down-regulation of BDNF. However, these authors transected the right sciatic nerve at its origin from the L4 and L5 spinal nerves and used *in situ* hybridisation to quantify mRNA levels and intrathecal administration of NT-3 at different dose, which may activate different intracellular pathways and produce different effects (Watson et al., 2001)(see 1.2.12), whereas I used real-time quantitative PCR and applied NT-3 systemically.

Only a few studies have examined the interactions between members of neurotrophin family. An *in vitro* study, using transfected PC12 cells, which express trkA and $p75^{NTR}$, but not trkB or trkC, has shown that exogenous NGF can induce release of BDNF, NT-3 and NT-4/5, mediated by both trkA and $p75^{NTR}$; exogenous BDNF induces NT-3 release, via $p75^{NTR}$; and exogenous NT-3 induced secretion of BDNF and NT-3 itself, probably via $p75^{NTR}$ but not trkA (Kruttgen et al., 1998). With regard to the possible mechanisms responsible for the neurotrophin-induced neurotrophin release, increasing intracellular calcium mediated by p75 and/or trk receptors through PLC-γ and PI-3 kinase pathways were proposed; an indirect pathway suggested triggering of neurotransmitter release which then causes depolarization and neurotrophin release (Kruttgen et al., 1998). However, the question of whether NT-3 can affect the secretion of NGF *in vitro* was not addressed in that study, although an *in vivo* study also described an up-regulation of BDNF mRNA expression in response to intrathecal administration of NGF, mostly in trkA-expressing DRG neurons (Michael et al., 1997).

On the basis of my results, I suggest that the NT-3-induced early up-regulation of NGF and BDNF levels in this study could be secondary to the activation of trkC or $p75^{NTR}$ signalling in DRG neurons and satellite cells. Signal transduction through the increased cross-activation between NT-3 and trkA or trkB in injured state due to the depressed $p75^{NTR}$ mRNA expression cannot be excluded. The NT-3-stimulated up-regulation of NGF mRNA in axotomised ganglia may have an effect on pain processing, and the up-regulation of BDNF may be anterogradely transported to dorsal horn and modulate the activity of trkB-expressing neurons in the spinal cord (Michael et al., 1997; McMahon, 1996; Woolf, 1996). However, systemic NT-3 treatment did not seem to exacerbate autonomy or change

the pain behaviour of rats, when compared with vehicle treatment. The increased BDNF may act through $p75^{NTR}$ on Schwann cells to stimulate remyelination (Notterpek, 2003), and the further up-regulated NGF may be involved in the formation of sympathetic nerve sproutings in the axotomised DRG (Zhou et al., 1999b; Deng et al., 2000).

4.3.6 Effect of systemic NT-3 administration on mRNA expression of neurotrophin receptors

It has been shown that 7-day intrathecal NT-3 administration caused the down-regulation of trkA but not trkC in uninjured DRG, using *in situ* hybridization (Gratto and Verge, 2003). Although these results are not really comparable to injury studies, they were to some degree in agree with my results of trkA and trkC expression in the contralateral ganglia of NT-3 treated rats at earlier time points.

The expression of neurotrophin receptors in axotomised ganglia after NT-3 administration has never been examined *in vivo* before. The present study showed that systemic NT-3 has effects on the expression of mRNA for all trk receptors. The decrease of trkA mRNA expression in axotomised DRG was counteracted by NT-3 from two weeks after injury; trkB mRNA was up-regulated by NT-3 treatment from 2 weeks following injury; the increase in mRNA expression of trkC in axotomised ganglia was totally counteracted by exogenous NT-3 until one week and then remained only slowly elevated but still at levels lower than vehicle-treated DRG for another week. At the last time point examined, 4 weeks, all three trk receptors were up-regulated by NT-3 administration, compared with either unoperated levels or vehicle-treated ganglia.

Because the early decrease of trkA mRNA in vehicle-treated axotomised ganglia is at least partly due to a decrease of target-derived NGF (as discussed in 4.3.2), the rebound of trkA transcripts in NT-3-treated ganglia, which were expressed in many neurons of all sizes, from 2 weeks implies that exogenous NT-3 may act directly via trkA or $p75^{NTR}$, or indirectly via other signalling pathways or by the up-regulation of NGF mRNA.

In a previous study using BDNF null mutant mice, it has been shown that endogenous BDNF is not required for the developmental induction of trkB expression or for the onset of BDNF dependence in sensory neurons (Huber et al., 2000). As BDNF is mainly synthesised in normal DRG and its expression increases in injured ganglia (Michael et al., 1999; Karchewski et al., 2002), the delayed decline of this receptor after axotomy suggests that BDNF itself does not regulate trkB mRNA expression. The increased expression of trkB mRNAby NT-3 treatment could be due to signal transduction initiated by NT-3 stimulation of trkB or $p75^{NTR}$.

The early counteraction of the increased expression of trkC mRNA by NT-3 treatment in injured DRG suggests that NT-3 from target organs is a major factor in regulating trkC expression in adult DRG. As systemic NT-3 administration has no effect on $p75^{NTR}$ mRNA expression in injured ganglia, in contrast to the complete recovery of $p75^{NTR}$ mRNA by intrathecal NGF infusion (Verge et al., 1992), it is assumed that target-derived NT-3 is not the main factor regulating p75 expression in adult DRG.

All together these results show that systemic NT-3 administration up-regulates mRNA expression of neurotrophins except NT-3 itself and counteracts, albeit only temporarily for trkC, the axotomy-induced changes of trk mRNA in DRG. NT-3 administration is likely to enhance the responsiveness of trkA- and trkB-expressing neurons

to their ligands by increasing the mRNA levels of both receptors and ligands in axotomised DRG.

NT-3 is known to be capable of activating all trk receptors (Cordon-Cardo et al., 1991; Lamballe et al., 1991; Soppet et al., 1991), although whether this happens *in vivo* is not clear. As the markedly decreased level of $p75^{NTR}$ mRNA after axotomy was proposed to result in at least partial loss of specificity to their ligands for trkA and trkB, these two trk receptors, even when down-regulated, may be more responsive to exogenous NT-3 than in intact state. In contrast, lower $p75^{NTR}$ co-expression with trkC may increase its specificity for NT-3 (Bibel et al., 1999; Vesa et al., 2000; Mischel et al., 2001). NT-3 at a normal physiological concentration has no effect on other trk receptors (Ip et al., 1993); however it may be possible for exogenous and injury-induced endogenous NT-3 to activate tyrosine phosphorylation via some or all trk receptors under these circumstances.

4.3.7 Developmental similarities

In order to ascertain whether the results of morphological and genetic study in axotomised DRG support the hypothesis of nerve cell replacement, I have compared them with known events during DRG development, although I am fully aware of the considerable differences between the two conditions.

During mouse development, DRG neurogenesis begins shortly before E10 with a burst between E12 and E13 (Lawson and Biscoe, 1979; Farinas et al., 1996); trk proteins start to be expressed concomitantly with neuronal differentiation (Klein et al., 1990b; Tessarollo et al., 1994). At E10.5-E11 in mice, when more than 60% of the DRG cells are

still at a precursor cell stage, most DRG neurons express trkC protein, whereas trkB is present in a modest subpopulation of cells and only low levels of trkA are present (Farinas et al., 1998). During that early stage, it has been suggested that NT-3 can directly activate trkB as well as trkC *in vivo* (Farinas et al., 1998). From E11-13.5, when trkA-expressing axons start penetrating into the proximal hindlimb and approach cutaneous targets, trkA expression increases steadily to appear in the majority of cells; on the other hand trkC decreases, to be expressed only in a subpopulation of DRG cells and persists into adulthood (White et al., 1996; Farinas et al., 1998). On the other hand, trkB expression undergoes no obvious changes during this period. In the present study, the mRNA expression of trk receptors during the 2 weeks after sciatic nerve axotomy parallels the events during embryonic neurogenesis, when trkA expression is low and most neurons express trkC.

In mice, NGF mRNA starts to be detectable in the surface ectoderm of the developing hindlimb at E11.5 and its expression increases from E13.5 to E15.5 when NGF is strongly expressed in the epithelium of the hindlimb epidermis and subjacent mesenchyme instead of the skin as a main source of synthesis in adult (Schecterson and Bothwell, 1992; White et al., 1996). NT-3 mRNA is expressed in mesenchymal cells in the dermamyotome immediately adjacent to the DRG and in muscle precursors in the proximal limb bud between E10 and E12 before their axons have reached their peripheral targets (Farinas et al., 1996; White et al., 1996). In my study, the NGF and NT-3 mRNA expression was increased in neurons and induced in satellite cells after axotomy, which is to some degree similar to the scenario during embryogenesis when the tissue adjacent to DRG releases neurotrophins and plays important roles.

These changes described may reflect an adaptive response to injury but whether the tissue would revert to a less differentiated state (de-differentiation) after peripheral nerve injury is questionable; increased local synthesis of neurotrophins or subsequent external stimuli (e.g. exogenous neurotrophins) may stimulate regeneration, or even neuronal replacement.

4.4 Gene expression profile in adult rat dorsal root ganglion after axotomy and systemic NT-3 administration

This section will discuss the data of microarray, which was performed to screen 1322 genes relevant to neurobiology in unoperated rats and at 2 weeks after mid-thigh sciatic nerve transection with and without systemic NT-3 treatment. The main emphasis will be on how axotomy and exogenous NT-3 regulate signalling pathways, growth associated proteins and other important genes and how this technique assists us to better understand the molecular mechanisms of morphological findings from axotomised ganglia.

4.4.1 Time point of microarray study and technical considerations

In this study, microarray was used to screen mRNA expression of more than one thousand genes. The transcription of genomic DNA into mRNA is the first step in the process of protein synthesis; alteration in mRNA expression reflects the intracellular responses to external stimuli, such as administration of neurotrophic factors and sciatic nerve injury. Although only the mRNA, which is an intermediate molecule in protein synthesis, can be studied, mRNAs for most genes are regarded as good indicators of

corresponding protein levels. However, in some conditions mRNA levels may not reflect the levels of protein, because regulation may occur at the post-transcription stage (Gygi et al., 1999).

There are mainly two types of array: oligonucleotide arrays and cDNA arrays. Each gene included in an oligonucleotide array is represented by one set of 16 different 'probe pairs.' Each probe pair consists of a 25-base-pair (bp) 'match' probe and a 25-bp 'mismatch' probe, in which the 13^{th} base does not match the target sequence. After hybridisation, the probe pair saturation is detected and analysed to generate a Detection *p*-value and assign a Present (P), Marginal (M) or Absent (A) call (http://www.affymetrix.com). Instead of using 25-bp probes, a cDNA array is produced by spotting PCR products of around 0.6-2.4kb, either full-length cDNAs or partially sequenced cDNAs. The disadvantage of cDNA arrays is that cDNA is deposited in a double-stranded form and tends to have intra-strand cross-link or some constraining contacts with the matrix (membrane or glass) along its length, which can be avoided in oligonucleotide arrays. One more advantage for using an oligonucleotide array is that each sample is hybridised onto a separate array, which allows independent repeated experiments with multiple samples without pairing or matching groups beforehand. However, using oligonucleotide arrays may have the disadvantages of the reduction of specificity and variations in melting temperature due to AT-CG composition (Duggan et al., 1999).

Although microarray accelerates the study of known or novel genes, it cannot totally replace other quantitative methods, including northern blots, RT-PCR, nuclease protection assays, and *in situ* hybridisation, which target on specific genes, and are able to quantify smaller changes in transcripts and confirm or extend microarray results. One more

limitation of this technique is that the starting amount and quality of total RNA is also an important factor. If microarray is to be applied to cases where only limited starting materials can be yielded, for example the LCM-selected cells, RNA amplification or multiple rounds of linear amplification based on cDNA synthesis and template-directed *in vitro* transcription might be required (Eberwine et al., 1992; Spirin et al., 1999). In addition, RNA was extracted from whole ganglia which contained different cell types including sensory neurons, satellite cells, Schwann cells, mast cells, fibroblasts, perineurial and endothelial cells; different regulation of genes in various cells should be considered.

To make it possible to compare data from different arrays that had been hybridised at the same time in this study, average intensities of gene expression were normalised (or scaled) to a value of 100. In other researchers' experience of using the array chips including more than thousands of genes in different experimental groups, only a small portion of present genes changed, being either up-regulated or down-regulated. Normalising (or scaling) the mean intensities in each array to a value of 100 for the majority of experiments seemed to be a reasonable and feasible method before further statistical analysis. Although a few potential "housekeeping control genes", including GAPDH, actin and hexokinase, are included in these arrays, it is not appropriate to use just one of them to normalise the data before confirming that a proposed " housekeeping gene" does not change under the experimental circumstances (sciatic nerve transection in my study) using other quantitative methods. In these microarray data, there was no significant difference in expression of GAPDH between the three groups, which is consistent with previous studies (Fan et al., 2001; Kim et al., 2001; Kubo et al., 2002; Xiao et al., 2002). These results support the

reliability of the data analysis of my real-time PCR study in section 2.4.10 in which GAPDH was used to normalise all the examined genes.

In my study, Rat Neurobiology U34 arrays from Affymetrix were used, which are a type of oligonucleotide array with a sophisticated internal control. 1322 sequences relevant to neurobiology research are included in each array chip. In view of the variability between animals, reproducibility and false positive detection, ganglia from 4 rats were pooled before hybridising onto an array and triplicate experiments in each group were performed. According to the data yielded from the study of neuronal number and apoptosis, which support the existence of newly-formed neurons in NT-3-treated axotomised DRG, and my data showing that systemic NT-3 can regulate the mRNA of all of the trk receptors, as well as that for NGF and BDNF in injured DRG, simple knowledge of the preferred pathway for NT-3 via trkC is not enough to explain the complexity of events triggered by nerve injury. In addition, my results also show that neurotrophin mRNAs were up-regulated, especially NGF, whose up-regulation lasted for more than 1 week, and some neurotrophic factors (such as glial-derived neurotrophic factor, transforming growth factor, ciliary neurotrophic factor and bFGF) have been reported to be up-regulated in axotomised DRG without treatment (Costigan et al., 2002; Xiao et al., 2002), finding out the signalling pathways which are regulated by axotomy may help to explain the mechanism of post-axotomy changes in ganglia. Moreover, these results may be derived from interactions of multiple pathways and proteins in DRG cells and neurons.

On the basis of these data I chose to examine gene expression patterns using the U34 Neurobiology to identify some of the genes implicated in development, regeneration and adaptive responses in axotomised DRG following vehicle or NT-3 treatment. The time

point of 14 days after sciatic nerve transection was chosen in this study because it is the time when neuronal apoptosis begins to be observed and the changes of neurotrophin /receptor mRNA are significant.

The results show altered mRNA transcripts (>1.5-fold) of 118 genes, including known genes and ESTs, 2 weeks after mid-thigh sciatic nerve transection with vehicle administration compared to sham operation. Microarray analysis of the alterations in gene expression caused by the injury demonstrates the response in DRG cells, which include sensory neurons that have been deprived of part of the axon and peripheral synaptic contacts. Real-time quantitative PCR were used to confirm 7 genes and validated my choice of 1.5-fold change to be a significant change. On the basis of my above considerations, I am confident that the method I apply is appropriate and adequate to find out which genes are involved in the process leading to the morphological changes I observed, and provides reasonable explanations of the NT-3-stimulated effects in axotomised DRG.

4.4.2 Gene expression profile in adult rat DRG after axotomy and systemic NT-3 administration

Three chips were used for each group and cross-comparisons were made between all the chips generating nine pair-wise sets of data; the regulated genes were categorised into 9 classes and selected genes were discussed to link their function to the reaction of neurons in injured state or after systemic NT-3 administration.

4.4.2.1 Signal transduction and cell regulation gene expression

In this study, expression of a few genes of signalling pathway involved in regeneration and neural development was regulated by axotomy and NT-3 treatment.

Mothers against decapentapleic (dpp) (Mad) is a mediator of the signal transduction pathways of transforming growth factor beta (TGF-β) superfamily, which includes TGF-βs, activins and bone morphogenetic proteins (BMPs). Mad1 was reported to mediate BMP signalling (Hoodless et al., 1996), which instructs the maturation of neural crest-derived progenitor cells to neuronal lineage and regulate subsequent neuronal differentiation in peripheral nervous system (Shah et al., 1996; Zhang et al., 1998); BMP-2 signalling has been shown to induce trkC expression and NT-3 responsiveness in sympathetic neurons (Zhang et al., 1998). BMP receptors are expressed in adult DRG, mainly in large sensory neurons, which also express trkC (Zhang et al., 1998). The significant increase of Mad1 transcripts (4.50-fold) after axotomy in the present study follows the activation of this pathway after peripheral nerve transection and supports the idea that peripheral nerve transection initiates the processes associated with neural differentiation and up-regulates trkC expression in DRG. As a result, the axotomised DRG neurons are likely to become more responsive to either endogenous or exogenous NT-3. In addition, axotomy-induced increase of Mad1 was further up-regulated by NT-3 treatment, suggesting that the signalling pathways initiated by the activation of the BMP receptors were stimulated by NT-3 either directly or indirectly.

Noggin, which is widely distributed and secreted as a homodimeric glycoprotein, acts by binding and limiting BMP actions (Zimmerman et al., 1996), and plays an important role in neural tube development (McMahon et al., 1998). After sciatic nerve transection, Noggin mRNA was decreased by 1.89-fold, which would also facilitate BMP

signalling in DRG neurons in concert with the increased Mad1. NT-3 administration had no effect on the expression of this gene.

Janus protein tyrosine kinases (JAKs) are important components of signal transduction pathways activated by a variety of growth factors and most cytokines (Ihle et al., 1995). JAK signalling pathway has cross-talk with other neurotrophin-activated signalling pathways, such as MAP kinase pathway and PI-3 kinase pathway (Rane and Reddy, 2000). However, whether JAKs are implicated in signal transduction processes initiated by NT-3 or other neurotrophins or neurotrophic factors is not clear. The growth of regenerating axons in cultures of DRG neurons, 2 weeks after a "conditioning" lesion on the sciatic nerve, depends on the JAK signalling pathway instead of the MAP kinase and PI-3 kinase pathways, both of which are crucial during development (Liu and Snider, 2001). My data show increased JAK1 mRNA expression, which is consistent with its persistent up-regulation observed by Rane et al. (2000) in axotomised DRG, implying the importance of the JAK pathway in enabling neurons to survive the loss or depression of other signalling pathways (Rane and Reddy, 2000). The mRNA expression of JAK1 was not affected by NT-3 treatment.

At least three intracellular pathways are known to be important mediators of survival, differentiation and neurite growth of sensory neurons: the ras/raf/mitogen-activated protein (MAP) kinase pathway, the phosphatidylinositol 3'-kinase (PI-3) kinase/Akt pathways and the phospholipase C-γ (PLCγ) pathway after activation of trk receptors (Vetter et al., 1991; Obermeier et al., 1993; Baxter et al., 1995; Goldberg and Barres, 2000). In this study, none of the genes relevant to these pathways were significantly

modulated by axotomy with vehicle administration, in contrast to the activation of BMP signalling and JAK pathway.

In neurons, Ras GTPase-activating protein (SynGAP) is enriched at excitatory synapses in brain and may be coupled to NMDA receptor function, negatively regulating Ras activity and playing a role in synaptic plasticity (Kim et al., 2003). The SynGAP knockout mice have smaller brains and die perinatally, showing its importance during early postnatal development (Kim et al., 2003). In my study, the increased SynGAP transcripts after axotomy may result in the inactivation of Ras, which is downstream of the MAP kinase pathway and which is normally initiated by neurotrophins or Ca^{2+} (Fukunaga and Miyamoto, 1998), inhibiting signal transduction through this pathway in axotomised neurons. SynGAP mRNA was not regulated by NT-3 administration.

Interestingly, systemic NT-3 treatment for 2 weeks after axotomy up-regulated 3-fold PI-3 kinase regulatory subunit p85α mRNA levels, which controls sequential activation of PI-3 kinase (Jimenez et al., 2002). The cAMP response element binding protein (CREB), which is a known regulator of genes required for neuron survival and is involved in the PI-3 kinase and PLCγ pathways (Riccio et al., 1997; Johnson et al., 2000), was also increased by 1.7-fold after NT-3 administration, compared to vehicle-treated ganglia. *In vitro* studies showed that the transcription of *bcl-2*, which is known to suppress apoptosis, was induced in neurons through CREB by insulin-like growth factor-a (IGF-1), NGF and BDNF (Pugazhenthi et al., 1999; Riccio et al., 1999); whether NT-3 also regulates *bcl-2* through CREB and then promotes DRG neuronal survival is not known, but my morphological results implied that NT-3 had no anti-apoptotic effects *in vivo*. This result indicates that of the three main signalling pathways for NT-3, the PI-3 kinase

pathway is activated by systemic administration of this neurotrophin at 2 weeks and may play a crucial role in neuronal regeneration and replacement of dying neurons. PI-3 kinase pathway also affects calcium homeostasis in cells, which is critical to survival (Tong et al., 1996).

In addition, a few genes related to neuronal development were regulated by axotomy and/or NT-3 administration. VGF, a secretory peptide precursor, widely expressed by neurons and neuroendocrine cells throughout the nervous system, plays an important role in developing neurons when they complete migration and begin to differentiate, and is involved in energy metabolism in adult CNS. VGF synthesis in DRG was first observed at embryonic day 13.5, but its exact function in DRG is not known (Snyder et al., 1998; Salton et al., 2000). In this study, VGF increased by 3.4-fold after axotomy; however, 2-week NT-3 administration did not significantly regulate its expression in DRG as NGF did in PC12 cells and NT-3 did in primary culture of cortical or hippocampal neurons (Hawley et al., 1992; Bonni et al., 1995). Its cell type-restricted regulation of transcription may be an explanation.

Monocyte chemoattractant protein-1 (MCP-1) was observed to increase in experimental stroke models (Kim et al., 1995; Yamagami et al., 1999), and contributes to recruitment of neutrophils, lymphocytes and monocytes into the damaged tissue (Ousman and David, 2001). MCP-1 levels were significantly increased in ischaemic brain and were also involved in the migration of bone marrow stromal cells (MSCs) *in vitro* using a microchemotaxis chamber (Wang et al., 2002b). MCP-1 was also shown to inhibit neuronal and astrocyte apoptosis in the CNS (Eugenin et al., 2003). The 2.8-fold increase of MCP-1 expression in axotomised ganglia in my arrays supports the idea that MSCs can be

potentially attracted, migrate to injured DRG, and that this may be enhanced by MCP-1, and get ready for further signals (e.g. NT-3) to differentiate. MCP-1 up-regulation may also explain the lymphocyte and macrophage invasion of DRG after axotomy (Hu and McLachlan, 2002). However, NT-3 treatment partly counteracted the injury-induced increase of MCP-1.

Chemokines are a superfamily of chemoattractant proteins, mainly functioning in control of leukocyte trafficking, and recruitment and activation of inflammatory cells. Chemokine CX3C, which exists as either a membrane-anchored protein or a secreted chemokine, is expressed in neurons, microglia and astrocytes, together with its G-protein-coupled receptor CX3CR1, in brain (Mizuno et al., 2003). This chemokine participates in the attraction and activation of microglia after brain ischaemia (Tarozzo et al., 2002). Chemokine CX3C can provide neuroprotective effects to hippocampal neurons *in vitro* by activation of Akt/PI-3 kinase pathway (Meucci et al., 2000; Mizuno et al., 2003), whereas its distribution and function in DRG is not clear, except for being expressed in neonatal DRG neurons (Oh et al., 2001). In my work, the presence and levels of chemokine CX3C mRNA in unoperated DRG indicated its activity also in normal adult DRG; the NT-3-induced up-regulation of this chemokine raises the possibility that it is involved in the activation of the PI-3 kinase pathway as well as the recruitment of certain types of cells which may be important for neuroprotection or neuronal replacement.

Cytosolic retinol-binding protein (CRBP) is involved in the oxidation of retinol to retinal and retinal to retinoic acid (RA), which initiates the differentiation of neurons and glial cells and induces early apoptosis; RA also stimulates neurite outgrowth from DRG neurons during embryonic development via nuclear receptors and modulates gene

transcription (Quinn and De Boni, 1991; Ross et al., 2000; Sarkar and Sharma, 2002). In cell cultures of stem cells from adult rat hippocampus, exposure to RA caused cell division, initiation of differentiation into immature neurons and up-regulation of trkB, trkC and $p75^{NTR}$; subsequent application of either NGF, BDNF or NT-3 enhanced neuronal maturation (Takahashi et al., 1999). In this work, the 1.9-fold increase of CRBP-I after axotomy suggests that the retinoid signalling is activated in injured ganglia and may contribute to the post-axotomy regulation of trk receptors, especially the up-regulation of trkC, and the differentiation of cells into Schwann cells, satellite cells and neurons. Although NT-3 did not significantly affect the levels of CRBP-I transcripts, it is proposed that NT-3, or other NT-3-stimulated neurotrophins, may subsequently enhance any neuronal maturation by way of RA-dependent expression of the Trk receptors (Takahashi et al., 1999).

Interferon-gamma inducing factor (IGIF), also named interleukin-18 (IL-18), is a relatively newly found cytokine and has been shown to be induced in macrophages during axotomy-induced Wallerian degeneration to modulate immune reactions after peripheral axonal injury (Menge et al., 2001). The expression of IGIF/IL-18 was reported to be increased in RA-treated mouse embryonic stem cell culture during neural differentiation (Sarkar and Sharma, 2002). Its increased mRNA expression in injured DRG shown here, which was not affected by exogenous NT-3, may be implicated in the post-axotomy inflammatory reactions, apoptosis of neurons and satellite cells and neuronal differentiation.

4.4.2.2 Growth-associated proteins and growth factors

Axotomy induced the up-regulation of growth associated protein 43 (GAP-43) by 2.4-fold, bFGF by 2-fold, NT-3 by 2-fold, BDNF by 1.7-fold, insulin-like growth factor (IGF)-2 by 1.6-fold and insulin-like growth factor binding protein (IGFBP)-6 by 1.6-fold. NGF is not included in the design of these commercial arrays.

In previous studies, these neurotrophic factors and their receptors have been demonstrated to play critical roles during nervous system development, and increased expression of some of these factors in DRG after axotomy, including NGF, BDNF, NT-3 and FGF-2, have been reported in the present study using real-time PCR and in previous papers (Sebert and Shooter, 1993; Grothe et al., 1997; Michael et al., 1999; Zhou et al., 1999; Karchewski et al., 2002).

GAP-43, a rapidly transported axonal protein localised primarily in the axonal growth cone, is strongly expressed during axonal outgrowth period of embryonic development. Its up-regulation by trauma has been shown to correlate with substantial functional recovery after axonal injury (Skene and Willard, 1981; Katz et al., 1985; Hoffman, 1989; Gispen et al., 1991; Fu and Gordon, 1997); although the rats in my study did not get any functional recovery or any growth cones after sciatic nerve transection and ligation for 4 weeks, the marked elevation of GAP-43 mRNA found in them suggested the potential of axonal regeneration, in keeping with previous studies (Costigan et al., 2002; Xiao et al., 2002).

Insulin-like growth factor II (IGF-II) has been reported to be expressed by Schwann cells after injury and to be involved in Schwann cell proliferation during peripheral nerve

regeneration (Svenningsen and Kanje, 1996); its receptor is expressed in a subset of DRG neurons of all sizes (Hawkes and Kar, 2002). Insulin-like growth factor binding proteins (IGFBP) modulate IGF actions in an autocrine or paracrine setting (D'Ercole et al., 1996). The up-regulation of IGF-II and IGFBP-6 in axotomised ganglia in this study implies that they may contribute to neuronal and non-neuronal adaptive reactions after axotomy.

Up-regulation of these growth-associated factors and growth factos may participate in the protection of neurons from death, in nerve regeneration and in modulation of some receptors and ion channels after axotomy (Doster et al., 1991; Fu and Gordon, 1997; Sterne et al., 1997; Bennett et al., 1998; Boucher et al., 2000). This provides a possible explanation for the survival of the majority of axotomised DRG neurons after sciatic nerve transection. However, these factors may be expressed in Schwann cells and satellite cells.

The above axotomy-induced potentially neuroprotective or regenerative factors were not up- or down-regulated by systemic NT-3 treatment. The only factor that was up-regulated by NT-3 treatment in injured ganglia in this category is fibroblast growth factor-18 (FGF-18), which is expressed in brain neurons during postnatal development and stimulates formation of glial cells in CNS (Hoshikawa et al., 2002). It was shown to be a neuroprotective agent in brain (Ellsworth et al., 2003); however the distribution and function of FGF-18 in DRG has not been studied before.

4.4.2.3 Apoptosis-related genes

The period of neuronal apoptosis in DRG following sciatic nerve transection has been reported to continue for at least 6 months after injury (Groves et al., 1997; McKay Hart et al., 2002). Although neuronal apoptosis was observed in the present study, no genes

specifically implicated in apoptosis (bcl-2, bcl-x, BAD, Fas, Bax) were found to be altered in any arrays, except a 2.3-fold increase of calmodulin-dependent protein kinase II (CaMK2).

Calmodulin (CaM) is a Ca^{2+}-binding protein involved in a variety of cell functions; CaMK2 is one of the CaM-dependent enzymes, which is known to be activated by the injury-induced Ca^{2+} overload and mediates the depolarisation-induced neuronal cell death in primary cortical neuron culture (Takano et al., 2003). However, the role of CaMK2 in neuronal damage *in vivo* is controversial (Takano et al., 2003). All the apoptotic neurons were immunoreactive for active caspase-3 in sections stained for it, indicating that this protease cascade is the main pathway of axotomy-induced neuronal death. CaM/CaMK2 signalling pathway may to some extent regulate the axotomy-induced neuronal death in DRG.

As the RNA used in this study was prepared from the pooled L4 and L5 DRGs, the contribution from apoptotic cells, approximately 0.04-0.07% of all neurons at a time point, is likely to be below the threshold applied in this study (>1.5-fold change). Although a study showed that axotomy-induced cell death might be related to their low ratio of cell death repressor bcl-2 and bcl-x to cell death promotor Bax expression in small-sized DRG neurons (Gillardon et al., 1996), in my data there was no significant up- or down-regulation of any of these genes.

In the present study systemic NT-3 administration did not affect any of the genes relevant to apoptosis; this is consistent with the morphological findings that there was no significant difference in the apoptotic rate between any operated groups. My results

confirm that systemic NT-3 treatment seemed to provide no neuroprotective effect against apoptosis in axotomised ganglia.

4.4.2.4 Cytoskeleton

The decrease in neuronal diameter in axotomised ganglia suggests that genes relevant to cytoskeleton are affected by axotomy and/or NT-3 treatment. The three major components of neuronal cytoskeleton are: (1) actin, which is particularly important in the structure of the growth cone; (2) microtubules, which regulate the movement of the growth cone; (3) intermediate filaments such as neurofilament proteins. A common feature of both development and regeneration after axotomy is the up-regulation of actin and tubulin, a component of microtubules, and the down-regulation of neurofilament (Wong and Oblinger, 1990b; Moskowitz and Oblinger, 1995).

The increase in mRNA for cytoplasmic β-actin I observed after axotomy is consistent with the increase of β-actin protein expression in DRG neurons reported following sciatic nerve crush and transection (Lund et al., 2002). β-actin is involved in the transport of cytoplasmic vesicles and is a limiting factor in the speed of axonal outgrowth during regeneration (Lund et al., 2002). The rapid induction of the mRNA expression of β-actin under the stimulation by NGF in PC 12 cells *in vitro* has been considered one of the general early gene responses to growth or differentiation factors (Greenberg et al., 1985; Chao, 1992). My data showed that the expression of β-actin was further up-regulated by systemic NT-3 administration, suggesting that exogenous NT-3 may stimulate axon growth after axotomy (Young et al., 2001). In some studies, β-actin, proposed to be expressed consistently, is often used as a housekeeping gene to normalise mRNA expression of a

gene examined by RT-PCR (Yun et al., 2002; Gnoatto et al., 2003). On the basis of my findings, it is not appropriate to use β-actin as an internal control for the mRNA quantification, especially when it is used in studies of the development and regeneration of the nervous system.

Microtubule-associated protein 1B (MAP1B) is expressed at high levels during development of the nervous system and is localised primarily in neurons and growing axons; on the other hand, MAP1A is not expressed during embryogenesis (Fawcett et al., 1994). Both MAP1A and 1B are expressed in adult DRG neurons and their axons, and are involved in the dynamics of microtubules in intact and regenerating axons (Fawcett et al., 1994; Ma et al., 1999). In the present work, MAP 1B mRNA was increased after axotomy with a nearly complete counteraction by NT-3 treatment; MAP1A was down-regulated following axotomy, but was not affected by NT-3 treatment. The changes in mRNA encoding MAPs in axotomised DRG I found seem to recapitulate a developmental pattern, and this suggestion is supported by a previous report that the expression of MAP1B mRNA and protein are expressed in Schwann cells during both development and axonal regeneration (Ma et al., 1999). Taken together, the findings regarding β-actin and MAPs show that the injured DRG neurons try to regenerate axons, even with a tight ligation of the proximal nerve stump. The NT-3-regulated changes in their mRNA expression may induce the reorganization of the cytoskeleton to alter the speed of axonal growth and axonal transport velocity in regenerating neurons (see Young et al., 2001).

My results show a down-regulation of neurofilament protein light subunit (NF-L) and neurofilament protein heavy subunit (NF-H) mRNA expression after axotomy, which is compatible with previous observations after sciatic nerve injury (Wong and Oblinger,

1987, 1990a). Systemic NT-3 treatment provided no significant effects on the mRNA expression of these two neurofilaments. The down-regulation of neurofilaments is a general trend during both development and regeneration (Muma et al., 1990; Troy et al., 1990; Wong and Oblinger, 1990b). As neurofilament content is a major determinant of axonal calibre and perikaryal diameter (Friede and Samorajski, 1970; Lawson and Waddell, 1991), the decrease in NF-L and NF-H expression probably underlies the decreases in perikaryal volume of DRG neurons seen 2 and 4 weeks after axotomy in my study.

The regulation of MAPs and neurofilaments by axotomy supports the notion that developmental processes are to some degree being recruited after peripheral nerve injury. Another protein required during axonal development, GAP-43, was up-regulated in DRG after axotomy in this study, in keeping with results by others (Hoffman and Cleveland, 1988; Meiri et al., 1988).

The mRNA levels of glial fibrillary acidic protein (GFAP) alpha, which is a major glial cytoskeletal protein (intermediate filament), were increased by 3.5-fold after axotomy in the present study and were consistent with the activation and proliferation of satellite cells in PNS reported following injury (Woodham et al., 1989; Fenzi et al., 2001). In CNS, astrocytes are also activated by motor axon injury, resulting in molecular changes including increased levels of GFAP (see Aldskogius and Kozlova, 1998). Although the functional implications of the glial cell responses in injured ganglia are unclear, these findings support the possibility that there could be some intercellular neuron-glia signals playing a role in satellite cells in response to peripheral nerve injury. The activated satellite cells may modulate the attempts of injured DRG neurons to survive and achieve functional repair. Systemic NT-3 treatment in my study further up-regulated GFAP mRNA, indicating that

NT-3 may stimulate glial cell proliferation in axotomised ganglia. This possibility is supported by the presence of a number of trkC-expressing satellite cells in unoperated and injured DRG using *in situ* hybridisation.

4.4.2.5 Pain-related genes

Neuropathic pain is induced by injury to the nervous system. To understand the complex molecular pathophysiology of neuropathic pain following nerve trauma, previous studies have focused on a few known genes and signalling pathways in DRG neurons. Recently, cDNA microarrays were used to identify additional genes which may contribute to the generation and maintenance of neuropathic pain after sciatic nerve transection or spinal nerve ligation (Wang et al., 2002a; Xiao et al., 2002).

In my study, galanin and NPY are up-regulated after sciatic nerve transection; in contrast, somatostatin (SST), calcitonin gene-related peptide (CGRP), substance P, RET ligand 2 (RETL2), preprotachykinin (PPT) and nicotinic acetylcholine receptor mRNAs are down-regulated.

NPY, acting as a transmitter within the nervous system through G-protein-coupled receptors, showed a marked 55-fold increase after sciatic nerve transection, which is consistent with previous reports (Wakisaka et al., 1991; Sterne et al., 1998). Systemic NT-3 treatment for 2 weeks did not affect the mRNA expression of NPY, a result that contradicts the finding that exogenous NT- 3 delivered by grafting impregnated fibronectin for 30 days attenuated the up-regulation of NPY (Sterne et al., 1998). However, the different methods of delivery, dose and duration of exposure to NT-3 used in the studies may results in activation of different pathways or other trk receptors (Watson et al., 2001)(see 1.2.12).

Increased amounts of the transcripts for NPY in injured ganglia may produce an anti-nociceptive effect (Hua et al., 1991; Bannon et al., 2000)

Galanin, a neuropeptide acting via G-protein-coupled receptors and ion channels, was up-regulated by 26-fold in axotomised DRG; similar results were reported before using immunohistochemistry, *in situ* hybridisation and microarray (Villar et al., 1989; Xiao et al., 2002). NT-3 administration made no significant effects on the mRNA expression of this neuropeptide. Galanin is involved in the normal growth and development of the nervous system (Ubink et al., 2003); increased transcripts for galanin may also produce an anti-nociceptive effect in the injured spinal cord (Bannon et al., 2000).

Somatostatin (SST) mRNA expression was reduced by 1.5-fold following axotomy and NT-3 treatment had no effect on this. SST, a non-opioid neuropeptide, is transiently expressed in most rat DRG neurons during the early development, but its mRNA is only detected again from postnatal week 2 in small DRG neurons. It is involved in pain transmission as an inhibitory neuromodulator (Maubert et al., 1994). In DRG neurons, SST was shown to be colocalised with RET, which is a functional receptor component of glial-derived neurotrophic factor (GDNF) (Baloh et al., 2000). The down-regulation of SST after axotomy could be rescued by GDNF administration to provide anti-inflammatory and anti-hyperalgesic effects (Bennett et al., 1998; Kashiba and Senba, 2000).

I found that RETL2 mRNA was decreased by 1.8-fold after axotomy, and that this was not affected by NT-3 administration. RETL2 interacts with the extracellular domain of RET, is a key component of the RET signalling pathway for GDNF and can only bind GDNF with high affinity in the presence of RET (Sanicola et al., 1997). Decreased expression of RETL2 in axotomised DRG may result in decreased GDNF signalling and be

related to the generation of neuropathic pain and neuronal death (Leclere et al., 1998). GDNF administration can reverse down-regulation of GDNF receptor components after axotomy (Bennett et al., 1998, 2000).

A 1.8-fold decrease in CGRPmRNA was shown by microarrays at 2 weeks after sciatic nerve transection; no effect of NT-3 on this decrease was observed which is in agreement with other work (Sterne et al., 1998). CGRP is a neuropeptide that is synthesised in approximately 50% of the small- and medium-size DRG neurons, especially trkA-expressing neurons (Averill et al., 1995), and is transported along the axon to the periphery and dorsal horn to modulate nociception (Ju et al., 1987; McNeill et al., 1988; Heppelmann and Pawlak, 1997). Depressed levels of CGRP mRNA were reported to last for up to 45 days after axotomy (Dumoulin et al., 1991; Sterne et al., 1998); it was suggested that this be due to decreased target-derived NGF (Verge et al., 1995; Shadiack et al., 2001); and NGF administration increases CGRP expression (McMahon et al., 1995).

My study showed that SP mRNA expression decreased by 1.6-fold after axotomy, as reported in a previous study at the same time point (Ma and Bisby, 1998); this decrease was also not affected by systemic NT-3, in keeping with previous observations. Similar to CGRP, SP is also released from both the peripheral and central terminals of nociceptive afferents into the periphery and dorsal horn to modulate nociception (Malmberg and Yaksh, 1992). The decline was probably due to decreased retrograde transport of NGF, and exogenous NGF was reported to up-regulate SP expression in DRG neurons *in vitro* (Lindsay and Harmar, 1989). Removal of NGF with antisera reduces SP and CGRP levels *in vivo* (Shadiack et al., 2001).

In my study, nicotinic acetylcholine receptor subunits were down-regulated following sciatic nerve transection, but were not further regulated by NT-3; in contrast, acetylcholine muscarinic receptor mRNA remained at 'unoperated' level after axotomy, and was up-regulated by NT-3 treatment. Normal rat DRG neurons are able to synthesise acetylcholine (Ach) and also express cholinergic receptors of both muscarinic and nicotinic type (Tata et al., 2000; Genzen et al., 2001). Muscarinic receptor subtypes are preferentially expressed in small-medium sensory neurons and satellite cells in DRG, indicating their association with nociception and possible cross-talk between neurons and satellite cells (Tata et al., 2000). Nicotinic receptors are also expressed by a majority of large DRG neurons and some small neurons (Genzen et al., 2001), both at cell soma and nerve terminals, to modulate somatic sensory transmission including nociception (Damaj et al., 1998). The regulation of these two types of Ach receptors shown here suggests a possible mechanism of neuropathic pain which may be regulated by exogenous NT-3.

In the current study, P2X2 receptor mRNA was increased by 1.5-fold in DRG following sciatic nerve transection. P2X2 receptor, which is a ligand-gated ion channel mainly expressed in small neurons of DRG, is known to mediate the action of extracellular ATP and plays a role, together with other P2X receptors, in nociceptive signalling (Ding et al., 2000). Its increase in injured DRG suggests extracellular ATP and this receptor could also be involved in the chronic neuropathic pain after nerve injury. NT-3 administration did not affect mRNA expression of this receptor.

In addition to muscarinic receptor, somatostatin receptor 4 (SSTR4) and calcitonin receptor-like receptor (CRLR) were the only two transcripts which were up-regulated by NT-3 administration in this category. SSTR4 is a subtype of the receptors for somatostatin,

and it is mainly expressed in the CNS, but also in the developing DRG from E12.5 to E16.5 in rat, suggesting a role during neurogenesis (Maubert et al., 1994). After brain injury, SSTR4 is up-regulated in glial cells and down-regulated in neurons (Schreff et al., 2000). Taken together the depressed SST and elevated SSTR4 levels indicate that somatostatin-induced signal transduction may play an important role in NT-3-treated DRG neurons to modulate pain transduction.

Calcitonin receptor-like receptor (CRLR), coupled with a receptor component protein (RCP) and a receptor activity-modifying protein (RAMP1), functions as a nociceptive CGRP receptor (Juaneda et al., 2000). CRLR has also been found to be present in bone marrow-derived macrophages, and interleukin-6 synthesis was stimulated by CGRP in these cells (Fernandez et al., 2001). Whether CRLR is expressed in DRG neurons or satellite cells or other cell type is unclear. The up-regulation of CRLR mRNA by NT-3 indicates either that systemic NT-3 administration may modulate nociception after axotomy, whist CGRP is still at a lower level than normal, or that it acts on macrophages in injured DRG to control cytokine secretion.

The complex regulation of pain-related genes after sciatic nerve transection, as discussed in the paragraphs above, shows that some of them may stimulate neuropathic pain whilst others may inhibit it. However, neuropathic pain mechanisms involve adaptive reactions in the periphery and also in the dorsal horn of spinal cord. As SP and CGRP expression are known to be regulated by NGF (Lindsay and Harmar, 1989), and NT-3 can activate trkA, NT-3 administration might be expected to up-regulate CGRP expression (Ip et al., 1993; Farinas et al., 1998). My results demonstrated that systemic NT-3 administration for 2 weeks did not significantly affect either CGRP or SP transcripts,

showing that NT-3 at this concentration does not produce similar effects on nociception as NGF, acting via the trkA receptor.

4.4.2.6 Proteins related to synaptic transmission

The expression of mRNA for the glutamate/aspartate transporter was increased by 1.9-fold following axotomy; but expression of mRNA for the glutamate receptor subunit 5-2 (GluR5-2) was decreased by 2.1-fold. NT-3 treatment up-regulated the mRNA encoding for the AMPA-selective glutamate receptor 1.9-fold in ipsilateral DRGs.

Glutamate is a major excitatory neurotransmitter in CNS, and can activate both ion-channel-forming (ionotropic) and G-protein-coupled (metabotropic) glutamate receptors (GluRs) to initiate its biological effects. The expression of these receptors at both protein and mRNA levels in DRG was investigated by Sato et al. (1993). Glutamate transporters, which are responsible for concentrating glutamate in synaptic vesicles and for controlling its concentration in the extracellular space after its release into the synaptic cleft, are expressed in large, CGRP-negative DRG neurons with transporter-containing nerve terminals in the spinal cord (Oliveira et al., 2003). The increased mRNA transcripts for glutamate transporter in injured DRG presented in this study imply that the release of glutamate from central terminals of glutamatergic DRG neurons may be involved in the adaptation of sensory processing after nerve injury. Although the distribution of glutamate transporter in large DRG neurons implies its colocalisation with trkC, NT-3 did not regulate the expression of this transporter.

In the present study the ligand-gated receptor GluR5 is down-regulated in the injured DRGs. GluR5 has been found to be expressed specifically in the presynaptic

terminals of C-fibres, especially in neurons that innervate the inner layer of lamina II in the dorsal spinal cord, and to have an inhibitory role in the regulation of neurotransmission in presynaptic cells (Hwang et al., 2001; Kerchner et al., 2001). It may be the case that the decreased mRNA expression of this receptor in my study could correlate with the disappearance of presynaptic inhibition of neurotransmission from primary sensory neurons, resulting in the increased activity of these neurons projecting to the dorsal horn of the spinal cord.

AMPA receptor is an inotropic receptor of glutamate; its mRNA and protein expression in DRG have been described in adult rat (Sato et al., 1993). The increased levels of AMPA have been linked to the long-term functional impairment after spinal cord injury (Grossman et al., 1999); however its function in normal and injured DRG is not clear. The NT-3-stimulated expression of AMPA shown here may alter the response of DRG neurons to glutamate if the up-regulation is in neurons. However, satellite cells have also been reported to express AMPA receptors (Tachibana et al., 1994).

My microarray data also showed that gamma-aminobutyric acid (GABA)-A receptor $\alpha 5$ subunit mRNA was up-regulated after axotomy, whereas GABA-B was down-regulated. Its receptors are divided into two classes, ionotropic (GABA-A) and metabotropic (GABA-B) receptors, both of which are expressed in DRG neurons (Maddox et al., 2004); GABA-B predominates within the dorsal horn of the spinal cord (Towers et al., 2000). Activation of presynaptic GABA-B receptor decreases the release of neuropeptides and excitatory amino acids; the activation of postsynaptic GABA-B receptors located on ascending second-order neurons may inhibit the response to noxious stimuli (see Towers et al., 2000). The combined effects of the injury-induced regulation of

these two receptors may lead to the change of presynaptic inhibition at the central terminal of the injured primary afferent and modulate the activation of dorsal horn neurons during nociception. The mRNA expression of GABA was not affected by systemic NT-3 administration.

Transferrin mRNA increased by 1.7-fold following axotomy in this study; NT-3 administration had no influence on its mRNA expression. Transferrin is the iron-transporting glycoprotein found in plasma; it can be detected as early as E18 in rat DRG neurons (Dion et al., 1988). The two main sources of transferrin in adult brain are oligodendrocytes and the choroid plexuses; CNS neurons also express transferrin mRNA *in vitro*; transferrin would bind with any extracellular iron to decrease oxidative injury to myelin (see Wagner et al., 2003). Increased synthesis of transferrin was seen in brain following a variety of brain injury (see Wagner et al., 2003).

Transferrin receptor mRNA in DRG was decreased after axotomy, and it was not regulated by NT-3 administration. Iron is required for many metabolic processes in the nervous system, including the formation of myelin, and the uptake, packaging and degradation of neurotransmitters (Beard et al., 1993). Iron-bound transferrin is taken up as an endosome into the cells expressing transferrin receptors. As damage to tissues and cells may result in release of iron, the decreased transcripts of transferrin receptor shown in the present study may provide some degree of neuroprotection.

4.4.2.7 Secreted and extracellular molecules

The transmission of nerve impulses across synapses is accomplished through the release of neurotransmitters from synaptic vesicles during calcium-regulated exocytosis.

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4.4.2.7 Secreted and extracellular molecules

The transmission of nerve impulses across synapses is accomplished through the release of neurotransmitters from synaptic vesicles during calcium-regulated exocytosis.

(Bark et al., 1995; Boschert et al., 1996). My study showed increased mRNA of synaptic vesicle protein (SV2), Synaptotagmin IV and syntaxin B, and a decreased abundance of SNAP25A at 2 weeks after axotomy. Systemic NT-3 administration for 2 weeks in axotomised DRG up-regulated syntaxin 4, and down-regulated SNAP-25A and 25B, ras-related mRNA (rab3), synaptojanin and synapsin Ia.

Rab2, 25 kDa synaptosomal associated protein (SNAP-25A), SNAP-25B, synapsin 2a and 2b, ras-related rab3, and neurexin-1β are the main components of synaptic secretory apparatus, and participate in vesicle docking, fusion, neurotransmitter release and neuroplasticity, such as neurite outgrowth and synaptogenesis (Grabs et al., 1996; Porton et al., 1999; Brunger, 2000).

Down-regulation of any components of synaptic secretory apparatus may hinder the functions relevant to neurotransmitters. For example, SNAP-25 has two isoforms: SNAP-25A, which is the major isoform expressed during embryogenesis; SNAP-25B, which increases during synaptogenesis in the post-natal period and predominates in the adult brain (Boschert et al., 1996). Decreased expression of SNAP-25A in injured ganglia may to some degree impair the release of neurotransmitter from central terminals of DRG neurons. NT-3 treatment further down-regulated the expression of mRNAs for SNAP-25A and SNAP-25B.

Synaptotagmin is a family of vesicle membrane proteins, involved in Ca2+-regulated exocytosis in neurons (Mikoshiba et al., 1999). Increased synaptotagmin IV in this study may decrease evoked synaptic transmission by decreasing efficiency at coupling Ca2+ to secretion (Littleton et al., 1999).

Two more synaptic vesicle-associated proteins were down-regulated by NT-3 administration: (1) Synaptojanin, which is a polyphosphoinositide phosphatase, playing

roles in clathrin-mediated synaptic vesicle endocytosis in CNS (Stenmark, 2000). (2) Synapsins, a family of phosphoproteins mainly expressed in neurons in CNS, which plays roles in controlling the number of vesicles available for release at the nerve terminus and neurite elongation and synapse formation during development (Ferreira and Rapoport, 2002).

Syntaxins are synaptic proteins implicated in docking of synaptic vesicles with the presynaptic cellular membrane (Rizo and Sudhof, 2002). Syntaxin 4 was up-regulated by NT-3 treatment in my study.

My data are consistent with the loss of synaptic terminals and reinforce the idea that nerve injury causes the down-regulation of genes associated with neurotransmission. The reduced levels of some synaptic vesicle-associated proteins after NT-3 treatment indicate that the docking and fusion of synaptic vesicles may be attenuated which may result in reduced signalling from injured neurons and act as a protective mechanism when axonal regeneration was impeded by permanent transection, and may represent increased resources going to axonal regeneration and less resources to synaptic transmission.

4.4.2.8 Others

A few genes not categorised in the above groups were also regulated by axotomy with or without NT-3 treatment.

In this study, neuronatin-alpha was increased by 1.9-fold following axotomy and was unaffected by NT-3 treatment. Neuronatin is a protein expressed primarily in the central and peripheral nervous systems with a proposed function in neuronal maturation and maintenance. In the fetus and neonatal stages, it is expressed at a higher level than in

adulthood and is down-regulated in PC12 cells upon NGF-induced differentiation (Wijnholds et al., 1995; Joseph et al., 1996). An *in vitro* study demonstrated that neuronatin protected differentiated PC12 cells against some toxic insults (Zheng et al., 2002). The increased transcripts of this gene suggest its implication on neuroprotection or regeneration after axotomy.

Sciatic transection also increased transcripts of tissue plasminogen activator (t-PA) mRNA, which was completely counteracted by systemic NT-3 treatment. The induction of this gene by sciatic nerve axotomy has been shown in another *in vivo* study (Siconolfi and Seeds, 2001). tPA, a secreted serine protease, is able to cleave plasminogen to produce the active protease plasmin (Vassalli et al., 1991), and to play important roles in tissue remodelling and dissolving the extracellular matrix to assist axonal regeneration (Siconolfi and Seeds, 2001). The mRNA for t-PA was up-regulated following axotomy, although axonal regeneration could not take place because of nerve transection and ligation. The prevention of up-regulation by NT-3 treatment implies that the involvement of NT-3-induced signalling in t-PA regulation and NT-3 may prevent the optimal environment for axonal regrowth to be established.

Neural adhesion molecule F3 mRNA was up-regulated in DRG after sciatic nerve transection, but was not further regulated by NT-3 treatment. Neural adhesion molecule F3 is an axonal adhesion molecule, which mediates bidirectional exchange of information between neurons and glial cells and stimulates Schwann cell migration during development and regeneration by binding to its receptors on Schwann cells (Xiao et al., 1997; Thomaidou et al., 2001). The change shown on the arrays is consistent with increased

neurite growth after axotomy regardless of the ligation of proximal stump of transected nerve.

Cortexin is expressed in adult DRG, and its levels do not change after axotomy, but are 2-fold up-regulated by NT-3 administration. Cortexin was shown to stimulate neurite growth in E10-11 chick DRG culture (Chalisova and Khavinson, 2000). These results further suggest that NT-3 may stimulate axonal regeneration.

In this study, heat shock protein 27 (HSP27) is up-regulated in axotomised DRG, but is unaffected by NT-3 administration. This is consistent with previous reports that HSP27, a chaperone protein thought to protect cellular macromolecules from damage, was up-regulated in DRG neurons within 48 hours after sciatic transection at mid-thigh level (Costigan et al., 1998; Lewis et al., 1999). HSP27 was shown to protect neonatal DRG neurons from axotomy- or NGF withdrawal-induced apoptosis (Lewis et al., 1999; Wagstaff et al., 1999). My results of increased HSP27 mRNA expression for more than 2 weeks, when neurons were dying at a steady rate, suggest that it may provide some protective effects and explain the survival of most DRG neurons after axotomy. NT-3 treatment provided no further effects on HSP27 mRNA expression, further indicating a lack of neuroprotective effects.

4.4.3 Summary

The results obtained show that sciatic nerve transection altered the expression of 88 genes and 30 ESTs in the DRG at 2 weeks following injury. Systemic NT-3 administration for 2 weeks after axotomy up- and down-regulated 33 genes and 11 ESTs. The changes in

the expression of these genes give some general insights into the events that occur at 2 weeks following peripheral injury.

In vehicle-treated axotomised DRG, BMP and JAK signalling pathways are activated. The up-regulated growth-associated proteins, transferrin, neuronatin and HSP27, and decreased transferrin receptor mRNA may provide neuroprotective functions. The changes of cytoskeleton proteins and up-regulated neural adhesion molecule F3 may reflect the continuing attempts of neurites to grow despite the presence of a tightly ligated transected stump. Neurotransmission from central terminals of DRG neurons tends to be impaired. The increased levels of MCP-1, NGF, CRBP, IL-18 could be relevant to the neuronal lineage commitment of progenitor cells, which are a potential source of replacing cells and neurons (Corti et al., 2002).

Systemic NT-3 treatment is likely to act through PI-3 kinase and BMP pathways. The proposed complex interactions between these signal transduction pathways may be involved in the pathological changes in DRG neurons after axotomy, and any neuronal replacement mechanism stimulated by NT-3 administration. By up-regulating β-actin and cortexin, NT-3 appears to be capable of further stimulating axonal regeneration after axotomy. Satellite cells in injured ganglia appear to be activated by exogenous NT-3, resulting in increased levels of mRNA for GFAP.

CHAPTER 5: CONCLUSION

This study used an animal model to investigate the morphological and molecular events following peripheral nerve injury, and first time to examine the effects of systemic NT-3 administration on neuron number, neuronal apoptosis, expression of neurotrophins, neurotrophin receptors and neurofilaments, and the gene expression profile in axotomised DRG. NT-3 was suggested to support survival of embryonic sensory neurons via trkA or trkB signalling rather than through its preferred receptor trkC; therefore NT-3 treatment may produce broader effects than any other single neurotrophin.

My results show that a shift to the left in neuronal size distribution (atrophy) with no significant neuronal loss is evident at 2 weeks after mid-thigh transection and ligation of sciatic nerve, when 0.057% of DRG neurons were observed to be apoptotic and were also activated caspase-3-immunoreactive; at 4 weeks following axotomy, when the incidence of neuronal apoptosis was around 0.04-0.059%, around 16% DRG neurons appeared to be lost in the ipsilateral ganglia. Furthermore, some small-sized (mean diameter: 20μm) nestin-immunoreactive neuron-like cells appeared in the injured ganglia at 4 weeks, which were not seen in any sections from normal or contralateral ganglia, showing the appearance of a marker for neuronal or glial progenitors.

Systemic administration of NT-3 for 4 weeks via subcutaneous micro-osmotic pump at the dose of 1.25 and 5 mg/4 weeks is effective in preserving neuron numbers after axotomy, but it does not prevent or delay neuronal apoptosis; the incidence of nestin-expressing cells in injured DRG was further increased to 3.89 % by systemic NT-3 treatment for 4 weeks. I suggest that the preservation of neuronal numbers takes place through stimulated neurogenesis or neuronal maturation from either the precursor cells in adult DRG or bone marrow, and the nestin-immunoreactive cells are candidates for the

replaced neurons. Neuronal precursors have been obtained from adult mouse DRG by Namaka et al. (2001) and found to be capable of differentiating into neurons *in vitro* when exposed to neurotrophic factors, including NT-3. However, our previous study, showing that BrdU labelling of few proliferating cells in DRG after sciatic nerve crush, in which model neuronal replacement was also proposed, suggested that cell proliferation was insufficient to explain the neuronal replacement. New neuron formation from neuronal precursor cells which may undertake the pathway towards final maturation/ differentiation when stimulated by NT-3 is preferred.

Another finding by real-time quantitative PCR and *in situ* hybridisation is that the mRNA expression of neurotrophins in DRG neurons and satellite cells increases in response to peripheral axotomy, suggesting that the up-regulation of endogenous neurotrophins may at least in part replace the interrupted retrograde transport of periphery-synthesised neurotrophins after sciatic nerve transection. The existence of different intracellular signalling pathways for neurotrophins activated by axonal and perikaryal trk receptors may affect the compensatory functions of axotomy-stimulated neurotrophin synthesis in DRG cells and the effects of exogenous neurotrophins applied locally, intrathecally or systemiclly. The decreased expression of mRNAs for trkA and trkB, and the increased expression of trkC mRNA in neurons of all sizes in axotomised ganglia suggest a higher overall responsiveness of DRG neurons to NT-3 after axotomy, in contrast to a decreased responsiveness to NGF and BDNF, and could have synergistic effects on regeneration when administered with other neurotrophins. Moreover, systemic NT-3 administration is demonstrated to increase trkA and trkB responsiveness in axotomised neurons by up-regulating both these receptors and their ligands, at various time points.

Although systemic NT-3 administration alone has successfully prevented axotomy-induced neuronal loss in the present study, combinations of neurotrophic factors, which act through the up-regulated receptors, may appear promising. Different neurotrophic factors might selectively enhance the protective or reparative mechanisms, act at different stages of the degenerative process or influence distinct subpopulations of DRG sensory neurons or non-neuronal cells.

My subsequent microarray study revealed that BMP and JAK signalling pathways, instead of MAP kinase, PI-3 kinase and PLCγ pathways for neurotrophins, are activated in axotomised DRG, suggesting the importance of other neurotrophic factors in post-axotomy adaptive reaction of DRG cells in addition to the locally synthesised neurotrophins. Systemic NT-3 treatment activates signal transduction through PI-3 kinase and BMP pathways, highlighting the involvement of these two pathways directly or indirectly in the effects of NT-3 treatment on neuronal replacement in DRG and regulation of the expression of associated genes after sciatic nerve transection. Combining the findings of the appearance of 1.6% small nestin-immunoreactive neuron-like cells in the axotomised ganglia and the up-regulation of a few genes relevant to neuronal differentiation after axotomy, I suggest that a neuronal replacement mechanism is initiated 2 weeks after axotomy in untreated animals and that this process can be further stimulated by systemic NT-3 treatment, leading to the final differentiation of neurons. A more unexpected influence of NT-3 on genes controlling various cellular functions, including pain-related genes, growth factors, secreted and extracellular molecules and cytoskelton, was found in this study. These show that developmental processes are to some degree being recruited after peripheral nerve injury, and NT-3 appears to be capable of further stimulating axonal

regeneration after nerve injury, of activating satellite cells in injured ganglia, and of attenuating the docking and fusion of synaptic vesicles as a protective mechanism. Examining a large number of genes simultaneously by microarray analysis is clearly an important step in elucidating their functional role and provides a good opportunity to serve as a baseline for evaluating the efficacy of treatments aimed at regulating the injury-related changes in gene expression. However, it should be considered that the other cells in addition to neurons, such as satellite cells, Schwann cells, fibroblasts and blood vessels, may also play a role in the changes of gene expression detected in this study. Further studies will address the functional properties of these genes, which will clarify the pathways involved in nerve regeneration and neuronal replacement and find effective therapies to ameliorate the effects of peripheral nerve injury and stimulate neurogenesis or inhibit neurogenesis.

It is still an open question whether there are either stem cells or any postmitotic cells in adult DRG which can contribute to the neuronal replacement which appears to take place as my results suggest in injured states after systemic NT-3 administration. Detection of BrdU incorporation in combination with sequential markers for progenitor cells and new neurons or using retroviral infection, which can only integrate into dividing cells, will help to understand the characteristics of these new neurons. Moreover, transplantation of green fluorescent protein (GFP)-expressing bone marrow into mice or rats before carrying out sciatic nerve transection and systemic NT-3 administration will help to confirm and identify the origins of these new neurons in the future.

Appendices

Appendix 1: Estimated numbers of neurons and apoptotic neurons in the ipsi- and contralateral L4 and L5 DRGs after transection and ligation of the right sciatic nerve with no treatment, systemic saline administration or systemic NT-3 administration , using a physical disector technique and morphological criteria.

Time/treatment / Animal	Neurons in left L4/L5 ganglia	Neurons in right L4/L5 ganglia	Mean R:L ratio in neuronal number (± SD)	Number of apoptotic neurons in ipsi-lateral L4/L5
Unoperated				
3486	29062	29669	1.021	0
3534	25913	28399	1.100	0
3535	25048	25877	1.033	0
3536	22499	24354	1.082	0
4025	27394	31321	1.143	0
4119	26072	27180	1.042	0
Mean ± S.E.M.	25998 ± 904	27800 ± 1036	1.070 ± 0.019	0
2 weeks /vehicle				
3963	24423	26187	1.072	16
3974	27970	26142	0.935	19
3975	26802	30558	1.140	20
3976	28107	25623	0.912	7
Mean ± S.E.M.	26826 ± 853	27128 ± 1151	1.015 ± 0.055	15.5 ± 3.0
2weeks /0.625mg NT-3				
3959	30920	28240	0.913	2
3960	30462	26250	0.862	22
3961	25474	27832	1.093	28
3964	33723	28817	0.855	28
3965	26094	25309	0.967	17
Mean ± S.E.M.	29335 ± 1556	27290 ± 653	0.938 ± 0.044	19.4 ± 4.8
4 weeks /axotomy only				
3420	34410	29035	0.843	18
3419	25994	25860	0.995	24
3142	28744	24700	0.859	10
3141	27961	26669	0.954	10
3418	27769	23709	0.854	18
Mean ± S.E.M.	28976 ± 1431	25995 ± 912	0.898 ± 0.030	16.0 ± 2.7
4 weeks /vehicle				
3698	26266	23380	0.890	13
3703	26048	26280	1.009	23
3704	24263	24580	1.013	14

3705	25097	20070	0.800	3
3706	27934	23728	0.849	19
Mean ± S.E.M.	25922 ± 617	23608 ± 1017	0.912 ± 0.043	14.4 ± 3.4
4 weeks /1.25mg NT-3				
3699	25019	27310	1.092	7
3700	25070	27437	1.094	15
3701	27608	25549	0.925	21
3702	28622	25552	0.893	4
3697	25617	27939	1.091	6
Mean ± S.E.M.	26387 ± 731	26757 ± 504	1.019 ± 0.045	10.6 ± 3.2
4 weeks /5mg NT-3				
4043	28485	29103	1.022	12
4090	29673	32221	1.086	8
4091	27824	26262	0.944	22
4092	24413	27184	1.114	10
Mean ± S.E.M.	27599 ± 1129	28693 ± 1317	1.042 ± 0.038	13.0 ± 3.1

Appendix 2: Total number of neurons measured in L4&L5 DRG.

Unoperated	3535		3536		3707	
	L	R	L	R	L	R
Total	598	619	614	620	606	658
2 week axotomy + vehicle	3974		3975		3976	
	L	R	L	R	L	R
Total	640	620	659	655	647	583
2 week axotomy + NT-3 (1.25mg/4 weeks)	3961		3964		3965	
	L	R	L	R	L	R
Total	644	678	650	626	637	664
4 week axotomy + vehicle	3698		3703		3705	
	L	R	L	R	L	R
Total	601	618	595	625	689	657
4 week axotomy + NT-3 (1.25mg/4 weeks)	3697		3699		3701	
	L	R	L	R	L	R
Total	628	621	662	607	624	622
4 week axotomy + NT-3 (5mg/4 weeks)	4043		4091		4092	
	L	R	L	R	L	R
Total	553	597	661	487	625	654

Appendix 3: Profiles of neuronal size— Unoperated

	1		2		3	
Animal	3536		3707		3535	
%	L	R	L	R	L	R
<10	0	0	0	0	0	0
10_12	0	0	0	0	0	0
12_14	0	0	0.165017	0	0.167224	0
14_16	0.325733	0.322581	0.660066	1.367781	0.167224	0
16_18	1.954397	2.419355	2.145215	2.431611	0.334448	1.615509
18_20	5.04886	4.032258	4.290429	6.382979	2.341137	1.938611
20_22	8.143322	9.032258	9.570957	8.81459	5.518395	4.038772
22_24	12.37785	11.93548	10.39604	11.39818	7.525084	6.623586
24_26	13.35505	14.51613	12.0462	11.39818	11.53846	11.14701
26_28	11.07492	11.12903	11.71617	10.94225	13.8796	12.27787
28_30	9.446254	7.903226	9.570957	7.294833	10.53512	10.17771
30_32	6.026059	6.451613	6.765677	5.6231	7.023411	9.208401
32_34	4.723127	3.548387	4.125413	5.015198	6.020067	6.300485
34_36	3.745928	3.064516	3.30033	4.103343	5.016722	2.261712
36_38	2.28013	5.16129	2.805281	3.951368	3.010033	3.392569
38_40	2.28013	3.709677	5.280528	5.167173	2.675585	4.523425
40_42	2.605863	3.064516	2.145215	4.863222	4.180602	5.169628
42_44	2.931596	3.870968	3.30033	2.735562	3.344482	2.584814
44_46	3.420195	3.225806	3.30033	2.12766	2.842809	3.877221
46_48	2.931596	2.258065	1.485149	2.735562	3.344482	3.231018
48_50	2.76873	1.774194	2.475248	1.519757	1.839465	3.392569
50_52	0.977199	0.645161	1.980198	0.759878	1.672241	3.71567
52_54	1.302932	0.645161	0.49505	0.303951	1.839465	1.77706
54_56	0.814332	0.483871	1.485149	0.303951	1.839465	1.453958
56_58	0.814332	0.483871	0.330033	0.455927	1.505017	0.646204
58_60	0.488599	0.322581	0.165017	0.151976	1.337793	0.323102
>60	0.162866	0	0	0.151976	0.501672	0.323102
No. of neurons examined	606	658	614	620	598	619

Appendix 4: Profiles of neuronal size— 2 week axotomy + vehicle

	1		2		3	
Animal	3975		3976		3974	
%	L	R	L	R	L	R
<10	0	0	0	0	0	0
10_12	0.151745	0	0	0.171527	0	0.16129
12_14	0.151745	0.763359	0.618238	0	0	0
14_16	1.669196	3.358779	0.927357	3.087479	0.3125	1.290323
16_18	2.579666	6.259542	3.554869	7.718696	2.03125	5.967742
18_20	6.980273	13.43511	5.873261	11.14923	4.375	10.80645
20_22	11.22914	14.65649	13.60124	13.37907	7.03125	13.22581
22_24	14.56753	9.312977	12.36476	8.747856	12.03125	9.516129
24_26	12.13961	8.854962	15.76507	5.317324	12.03125	9.677419
26_28	11.38088	7.022901	10.20093	8.404803	13.59375	8.387097
28_30	7.435508	5.496183	6.33694	5.831904	8.28125	7.096774
30_32	4.855842	3.053435	3.554869	4.631218	5.78125	4.83871
32_34	2.427921	5.038168	3.400309	3.945111	4.21875	5.483871
34_36	3.490137	5.648855	2.782071	5.145798	2.65625	3.548387
36_38	1.972686	4.427481	2.009274	4.459691	3.125	3.548387
38_40	2.427921	3.51145	2.318393	4.459691	2.96875	4.677419
40_42	3.490137	3.816794	2.009274	4.116638	3.75	3.387097
42_44	2.731411	1.832061	3.400309	2.915952	3.59375	2.419355
44_46	3.186646	1.984733	2.163833	2.058319	3.28125	2.096774
46_48	2.427921	0.763359	3.24575	1.543739	2.96875	1.451613
48_50	1.972686	0.305344	2.009274	1.200686	1.40625	0.806452
50_52	1.062215	0.305344	1.700155	0.857633	1.5625	0.967742
52_54	0.91047	0	1.081917	0.343053	1.25	0.322581
54_56	0.151745	0	0.463679	0	1.5625	0.322581
56_58	0.30349	0.152672	0.309119	0.171527	1.25	0
58_60	0.30349	0	0.15456	0.171527	0.46875	0
>60	0	0	0.15456	0.171527	0.46875	0
No. of neurons examined	640	620	659	655	647	583

Appendix 5: Profiles of neuronal size— 4 week axotomy + vehicle

	1		2		3	
Animal	3698		3705		3703	
%	L	R	L	R	L	R
<10	0	0	0	0	0	0
10_12	0	0	0	0	0	0
12_14	0	0.161812	0.145138	0	0.168067	0.64
14_16	0.998336	1.294498	1.161103	2.435312	0.168067	0.48
16_18	2.66223	2.10356	2.902758	4.718417	1.344538	2.72
18_20	4.991681	5.177994	4.644412	8.371385	3.02521	6.56
20_22	8.319468	7.928803	7.692308	12.48097	7.89916	8.96
22_24	11.14809	12.78317	9.869376	8.371385	10.58824	10.08
24_26	12.31281	9.546926	12.77213	12.32877	12.26891	7.2
26_28	12.81198	9.708738	9.143687	8.371385	13.44538	8.48
28_30	9.151414	7.443366	8.272859	6.240487	7.563025	8.96
30_32	5.15807	6.472492	6.095791	6.392694	6.386555	8.48
32_34	4.492512	4.20712	5.370102	5.936073	4.369748	9.6
34_36	3.161398	5.177994	4.499274	5.631659	3.02521	4.8
36_38	1.331115	4.045307	3.918723	5.175038	3.02521	5.76
38_40	2.66223	4.692557	4.644412	2.130898	2.857143	4.96
40_42	1.663894	4.20712	3.483309	3.348554	2.352941	4.32
42_44	1.830283	3.883495	3.773585	3.348554	3.529412	2.88
44_46	4.159734	4.045307	2.75762	2.130898	4.705882	1.6
46_48	3.161398	2.588997	3.193033	1.065449	2.857143	1.76
48_50	2.995008	1.941748	1.886792	0.761035	3.02521	0.16
50_52	2.163062	0.809061	1.886792	0.304414	2.857143	1.12
52_54	1.331115	0.647249	0.725689	0.152207	1.512605	0.16
54_56	1.331115	0.323625	0.580552	0.152207	2.016807	0.16
56_58	1.164725	0.323625	0.290276	0	0.504202	0.16
58_60	0.332779	0.323625	0.145138	0.152207	0.336134	0
>60	0.665557	0.161812	0.145138	0	0.168067	0
No. of neurons examined	601	618	689	657	595	625

Appendix 6: Profiles of neuronal size— 2 week axotomy + 0.625mg NT-3

	1		2		3	
Animal	3961		3965		3964	
%	L	R	L	R	L	R
<10	0	0	0	0	0	0
10_12	0	0	0	0	0	0
12_14	0	0	0	0.215517	0.181818	0.5703422
14_16	0.310559	0.737463	0.457666	2.155172	1.636364	2.0912547
16_18	0.931677	2.507375	2.517162	2.586207	3.636364	8.7452471
18_20	2.484472	6.489676	5.949657	9.051724	9.090909	11.596958
20_22	4.503106	12.24189	8.695652	12.06897	9.818182	9.5057034
22_24	9.627329	14.45428	9.610984	10.34483	12.18182	10.076045
24_26	14.59627	11.35693	9.839817	9.913793	13.09091	8.7452471
26_28	11.02484	7.522124	10.29748	7.112069	8	7.2243346
28_30	10.71429	5.752212	6.864989	7.758621	8	5.1330798
30_32	6.832298	3.244838	6.17849	5.603448	4.181818	4.9429657
32_34	4.192547	3.982301	5.491991	5.172414	2.545455	4.3726235
34_36	3.416149	2.359882	5.949657	4.310345	3.272727	5.5133079
36_38	1.708075	4.572271	4.576659	5.818966	2.909091	4.5627376
38_40	1.863354	3.834808	3.203661	3.663793	2.909091	4.3726235
40_42	3.726708	4.129794	5.034325	5.172414	4	2.6615969
42_44	3.416149	4.719764	3.203661	3.232759	2.545455	3.9923954
44_46	2.018634	3.687316	2.28833	3.448276	4.545455	1.7110266
46_48	2.950311	2.507375	3.661327	1.077586	1.818182	1.3307984
48_50	2.950311	2.212389	2.059497	0.431034	2.363636	0.9505703
50_52	3.416149	1.327434	1.372998	0.215517	1.272727	0.7604562
52_54	3.26087	1.917404	1.372998	0.431034	0.727273	0.3802281
54_56	2.484472	0.294985	0.686499	0	0.181818	0.3802281
56_58	1.552795	0	0.457666	0	0.727273	0.3802281
58_60	1.242236	0	0.228833	0	0	0
>60	0.776398	0.147493	0	0.215517	0.363636	0
No. of neurons examined	644	678	650	626	637	664

Appendix 7: Profiles of neuronal size— 4 week axotomy + 1.25mg NT-3

	1		2		3	
Animal	3697		3699		3701	
%	L	R	L	R	L	R
<10	0	0	0	0	0	0
10_12	0	0	0.151057	0	0	0
12_14	0.477707	0.575816	0.151057	0.329489	0.320513	0.643086
14_16	1.11465	1.535509	1.057402	1.482702	0.160256	2.250803
16_18	2.866242	3.262956	2.114804	4.283361	1.121795	3.054662
18_20	7.324841	6.333973	3.172205	7.413509	3.365385	7.71704
20_22	9.076433	11.70825	5.287009	8.566722	7.051282	13.66559
22_24	9.872611	14.39539	9.063444	11.69687	9.455128	13.82636
24_26	10.03185	8.253359	11.93353	10.54366	13.30128	8.199356
26_28	6.369427	9.213052	12.68882	7.248764	10.89744	9.163987
28_30	7.802548	8.06142	11.48036	7.413509	9.294872	4.662379
30_32	5.573248	3.838772	4.380665	7.248764	4.967949	5.948553
32_34	5.095541	3.838772	4.07855	6.095552	3.044872	4.662379
34_36	4.936306	3.262956	2.265861	5.107084	2.403846	4.662379
36_38	5.254777	3.454894	2.719033	3.459638	2.403846	4.823151
38_40	6.369427	3.646833	2.567976	6.425041	3.365385	4.823151
40_42	5.095541	4.03071	3.625378	2.635914	3.205128	3.376205
42_44	3.343949	3.454894	2.567976	3.789127	3.205128	2.411575
44_46	5.573248	4.606526	3.776435	1.812191	5.288462	1.286173
46_48	1.910828	2.495202	3.927492	1.647446	4.326923	1.286173
48_50	1.11465	2.111324	2.719033	0.823723	3.846154	1.607717
50_52	0.318471	0.767754	2.870091	0.494234	3.205128	1.286173
52_54	0.318471	0.383877	2.719033	0.494234	2.724359	0.482315
54_56	0.159236	0.191939	2.265861	0.494234	1.602564	0.1607717
56_58	0	0.191939	1.057402	0.164745	0.801282	0
58_60	0	0.383877	0.906344	0.329489	0.160256	0
>60	0	0	0.453172	0	0.480769	0
No. of neurons examined	628	621	662	607	624	622

Appendix 8: Profiles of neuronal size— axotomy + 5mg NT-3

	1		2		3	
Animal	4043		4092		4091	
%	L	R	L	R	L	R
<10	0	0	0	0.152905	0	0
10_12	0	0	0	0	0	0.205339
12_14	0.329489	0.167504	0.16	0.458716	0	0.616016
14_16	1.482702	1.005025	0.48	1.834862	0.453858	1.848049
16_18	4.283361	2.680067	3.68	7.492355	1.966717	6.570842
18_20	7.413509	6.030151	7.2	13.45566	4.689864	8.62423
20_22	8.566722	9.21273	11.84	14.52599	8.774584	10.47228
22_24	11.69687	12.0603	12.16	11.6208	13.01059	9.445585
24_26	10.54366	11.22278	14.88	8.103976	14.37216	9.856263
26_28	7.248764	9.380235	10.56	8.409786	10.28744	8.829569
28_30	7.413509	7.705193	8.48	7.186544	5.295008	6.365503
30_32	7.248764	6.197655	4.64	5.045872	5.748865	5.544148
32_34	6.095552	4.187605	2.88	2.905199	4.08472	4.312115
34_36	5.107084	4.355109	2.08	3.669725	4.08472	4.928131
36_38	3.459638	3.852596	3.36	4.740061	3.025719	5.954825
38_40	6.425041	2.680067	3.68	2.599388	4.84115	5.13347
40_42	2.635914	3.852596	3.52	2.752294	3.933434	2.258727
42_44	3.789127	3.685092	2.72	2.293578	2.723147	2.874743
44_46	1.812191	3.18258	2.72	1.834862	2.723147	1.64271
46_48	1.647446	4.187605	1.44	0.458716	3.782148	1.437372
48_50	0.823723	1.842546	1.76	0.152905	2.118003	1.232033
50_52	0.494234	1.172529	0.16	0.152905	1.664145	1.232033
52_54	0.494234	0.502513	0.48	0	1.059002	0.410678
54_56	0.494234	0.335008	0.64	0	0.75643	0
56_58	0.164745	0.335008	0.16	0.152905	0.302572	0
58_60	0.329489	0.167504	0.16	0	0.151286	0.205339
>60	0	0	0.16	0	0.151286	0
No. of neurons examined	553	597	661	587	625	654

Appendix 9: Percentage of immunoreactive neurons in the ipsi- and contralateral L4 and L5 DRGs after transection and ligation of the right sciatic nerve.

Time/treatment / Animal	% of nestin-IR neurons in ipsilateral L4/L5 ganglia	% of nestin-IR neurons in contralateral L4/L5 ganglia
Unoperated		
3535	0	0
3536	0	0
4025	0	0
4119	0	0
Mean ± S.E.M.	0	0
4 weeks /axotomy only		
4011	1.64	0
4012	1.5	0
4098	0.98	0
4099	2.27	0
Mean ± S.E.M.	1.60 ± 0.46	0
4 weeks /5mg NT-3		
4043	3.44	0
4090	2.54	0
4091	3.06	0
4092	5.48	0
Mean ± S.E.M.	3.73 ± 1.28	0
8 weeks /axotomy only		0
4020	0.45	0
4021	5.37	0
4094	3.08	0
4093	5.67	0
Mean ± S.E.M.	3.64 ± 1.21	0

Appendix 10: Nestin mRNA, examined by real-time PCR (copies/10000 copies GAPDH)

Group	Unoperated	1 day	1 week	2 weeks	4 weeks
Right DRG (vehicle)	0.30	0.92	1.54	2.74	6.30
	0.28	1.60	0.80	1.38	0.35
	0.26	1.35	1.18	2.59	0.66
	0.31	0.72	0.76	3.70	4.60
	0.25	0.78	0.52	3.01	4.10
Mean	0.28	1.07	0.96	2.68	3.20
SEM	0.01	0.17	0.18	0.38	1.16
Right DRG (NT-3)		0.80	0.33	5.84	0.21
		0.16	6.86	5.00	12.90
		1.69	1.74	4.66	6.87
		1.14	3.97	10.34	8.98
		1.27	1.98	6.77	4.35
Mean		1.01	2.98	6.52	6.66
SEM		0.26	1.13	1.02	2.14
Left DRG (vehicle)	0.36	0.38	0.25	0.40	0.21
	0.38	0.50	0.42	0.31	0.24
	0.26	0.25	0.28	0.56	0.36
	0.37	0.37	0.36	0.37	0.36
	0.29	0.27	0.18	0.24	0.31
Mean	0.33	0.35	0.30	0.38	0.30
SEM	0.02	0.04	0.04	0.05	0.03
Left DRG (NT-3)		0.42	0.18	0.51	0.27
		0.36	0.37	0.35	0.38
		0.33	0.50	0.30	0.25
		0.30	0.46	0.41	0.31
		0.26	0.37	0.37	0.42
Mean		0.33	0.38	0.39	0.33
SEM		0.03	0.06	0.04	0.03

Appendix 11: NGF mRNA, examined by real-time PCR (copies/10000 copies GAPDH)

Group	Unoperated	1 day	1 week	2 weeks	4 weeks
Right DRG (vehicle)	6.06	17.68	8.14	16.41	11.21
	2.80	13.17	9.77	7.73	7.34
	2.65	15.16	7.24	5.96	6.42
	2.20	21.07	9.67	4.33	10.76
	4.32	17.73	11.44	11.36	10.13
Mean	3.61	16.96	9.25	9.16	9.17
SEM	0.71	1.34	0.72	2.16	0.96
Right DRG (NT-3)		16.28	9.44	14.97	2.46
		6.27	16.68	16.65	21.32
		12.96	13.08	15.40	12.26
		7.08	18.87	9.23	14.39
		13.05	10.98	4.35	4.11
Mean		11.13	13.81	12.12	10.91
SEM		1.92	1.75	3.20	3.46
Left DRG (vehicle)	3.96	4.35	5.48	3.55	3.09
	3.48	6.01	7.26	3.64	3.28
	3.47	2.60	0.80	2.60	1.77
	2.90	1.45	2.19	1.39	1.20
		2.35	3.46	4.46	4.31
Mean	3.45	3.35	3.84	3.13	2.73
SEM	0.22	0.81	1.15	0.52	0.56
Left DRG (NT-3)		3.07	4.05	4.14	2.15
		0.80	2.08	5.76	4.57
		2.80	4.97	4.14	2.07
		1.77	4.80	2.13	5.56
		1.36	2.84	1.19	2.32
Mean		1.96	3.48	3.48	3.33
SEM		0.43	0.56	0.81	0.72

Appendix 12: BDNF mRNA, examined by real-time PCR (copies/10000 copies GAPDH)

Group	Unoperated	1 day	1 week	2 weeks	4 weeks
Right DRG (vehicle)	141.32	475.69	215.07	304.69	192.84
	156.66	592.61	154.56	316.73	240.54
	110.05	411.70	250.21	132.57	184.00
	163.49	535.05	543.91	188.82	154.07
	165.77	1077.07	331.23	125.02	210.78
Mean	147.46	618.42	299.00	213.57	196.45
SEM	10.28	118.50	67.57	41.21	14.34
Right DRG (NT-3)		1181.70	543.30	423.26	302.17
		827.22	1287.38	403.92	280.35
		1596.70	847.73	277.75	482.77
		542.11	965.11	459.55	200.19
		1264.70	1064.60	323.14	376.01
Mean		1082.49	941.62	377.52	328.30
SEM		182.29	123.02	33.49	47.71
Left DRG (vehicle)	115.48	286.74	337.00	237.85	209.96
	138.64	377.28	256.71	94.70	160.53
	130.63	262.12	108.79	145.27	131.13
	161.06	188.84	70.02	156.59	156.60
		314.96	245.06	156.00	130.79
Mean	136.46	285.99	203.52	158.08	157.80
SEM	9.50	30.97	49.58	22.97	14.44
Left DRG (NT-3)		71.67	91.86	165.11	63.89
		146.58	81.41	144.04	108.20
		143.06	103.90	153.43	49.14
		51.44	109.22	104.78	189.59
		119.51	153.65	140.20	89.66
Mean		106.45	108.01	141.51	100.10
SEM		19.18	12.39	10.14	24.59

Appendix 13: NT-3 mRNA, examined by real-time PCR (copies/10000 copies GAPDH)

Group	Unoperated	1 day	1 week	2 weeks	4 weeks
Right DRG (vehicle)	14.92	30.57	29.51	22.12	15.90
	9.85	19.78	11.93	4.68	8.92
	7.39	14.65	8.89	13.25	10.48
	9.98	23.46	7.19	7.15	17.88
	13.45		19.13	15.62	9.74
Mean	11.12	22.11	15.33	12.57	12.59
SEM	1.35	3.35	4.09	3.10	1.80
Right DRG (NT-3)		28.32	5.64	11.84	9.88
		21.75	8.29	19.00	13.87
		24.90	76.55	2.96	10.86
		31.32	37.85	16.24	17.38
		19.86	40.38	8.00	7.51
Mean		25.23	33.74	11.61	11.90
SEM		2.09	12.91	2.86	1.71
Left DRG (vehicle)	6.57	13.00	10.22	20.25	13.89
	8.34	18.24	8.44	4.11	10.27
	14.72	9.21	15.99	12.55	6.23
	19.57	10.37	19.25	9.19	15.00
		17.24	13.09	11.50	13.62
Mean	12.30	13.61	13.40	11.52	11.80
SEM	2.99	1.80	1.95	2.62	1.60
Left DRG (NT-3)		9.11	10.32	3.22	10.79
		14.32	5.23	5.89	13.43
		15.52	8.28	12.37	6.34
		5.92	15.90	15.27	14.38
		17.60	16.66	27.73	14.52
Mean		12.50	11.28	12.90	11.89
SEM		2.16	2.20	4.29	1.54

Appendix 14: TrkA mRNA, examined by real-time PCR (copies/10000 copies GAPDH)

Group	Unoperated	1 day	1 week	2 weeks	4 weeks
Right DRG (vehicle)	539.57	367.39	180.64	209.15	383.08
	458.67	469.45	339.72	332.35	270.42
	500.53	297.77	245.76	102.81	208.30
	520.59	301.49	315.72	256.64	302.96
	485.66	475.73	490.42	250.73	255.19
Mean	501.00	382.37	314.45	230.34	283.99
SEM	13.96	38.87	35.53	37.57	29.09
Right DRG (NT-3)		445.98	224.45	377.37	320.42
		404.57	182.71	321.93	1145.61
		164.96	271.64	492.98	1438.5
		443.56	242.9	435.98	960.43
		431.65	252.14	362.72	943.00
Mean		378.14	234.77	398.20	961.59
SEM		53.80	15.07	29.93	183.41
Left DRG (vehicle)	478.75	717.30	280.52	744.74	780.06
	546.51	647.69	506.14	426.51	680.99
	625.00	551.83	786.26	1107.44	604.03
	406.20	862.03	673.92	564.91	729.03
	329.03	809.65	1119.22	712.00	791.53
Mean	477.10	717.70	673.21	711.12	717.13
SEM	51.79	55.54	140.32	114.15	34.45
Left DRG (NT-3)		517.39	482.32	380.42	360.25
		229.37	219.82	500.98	220.51
		159.82	376.11	419.07	128.12
		338.00	408.98	198.35	706.56
		357.68	545.00	500.21	294.52
Mean		320.45	406.44	399.81	341.99
SEM		61.06	55.10	55.52	98.99

Appendix 15: TrkB mRNA, examined by real-time PCR (copies/10000 copies GAPDH)

Group	Unoperated	1 day	1 week	2 weeks	4 weeks
Right DRG (vehicle)	4.03	8.54	2.52	2.28	4.22
	8.60	3.23	3.8	1.30	4.33
	2.63	9.58	3.04	1.08	5.19
	3.12	3.39	1.70	0.88	6.56
	5.87	8.19	5.34	1.69	5.63
Mean	4.84	6.59	3.29	1.45	5.19
SEM	1.08	1.36	0.62	0.25	0.43
Right DRG (NT-3)		6.83	3.63	23.05	23.77
		2.30	2.06	17.96	21.69
		5.34	5.15	6.61	11.48
		8.82	4.99	16.01	22.58
		7.19	4.67	13.50	15.13
Mean		6.09	4.10	15.43	18.93
SEM		1.10	0.57	2.70	2.39
Left DRG (vehicle)	3.59	5.27	4.08	5.14	6.10
	6.28	2.10	2.35	6.61	10.19
	5.24	12.95	2.66	6.91	10.97
	5.50	4.73	2.93	6.54	11.20
		1.70	2.56	8.23	2.27
Mean	5.12	5.37	2.91	6.69	8.15
SEM	0.57	2.03	0.30	0.49	1.74
Left DRG (NT-3)		14.83	7.31	9.79	5.64
		12.33	21.71	8.79	21.69
		2.81	12.85	9.23	26.53
		8.99	15.02	24.82	14.41
		10.32	18.07	35.72	21.03
Mean		9.85	14.98	17.67	17.85
SEM		2.02	2.43	5.43	3.61

Appendix 16: TrkC mRNA, examined by real-time PCR (copies/10000 copies GAPDH)

Group	Unoperated	1 day	1 week	2 weeks	4 weeks
Right DRG (vehicle)	126.61	91.29	526.69	474.48	181.56
	61.53	473.75	388.30	445.88	137.95
	66.68	586.65	141.63	372.58	127.41
	108.32	536.28	677.51	327.72	260.72
	106.56	1031.52	155.33	465.92	207.1
Mean	93.94	543.90	377.89	417.32	182.95
SEM	12.70	149.90	104.25	28.70	24.23
Right DRG (NT-3)		116.97	252.99	260.96	390.83
		32.07	147.17	348.66	355.24
		149.30	249.76	256.95	257.20
		59.72	126.85	364.95	142.76
		116.15	227.45	336.33	338.35
Mean		94.84	200.84	313.57	296.88
SEM		21.31	26.62	22.76	44.32
Left DRG (vehicle)	109.91	305.42	390.20	154.24	173.10
	51.51	389.30	300.42	266.44	266.59
	90.46	358.42	127.65	241.77	197.87
	116.06	290.45	182.49	39.41	249.35
		186.96	103.39	210.83	212.31
Mean	91.99	306.28	177.16	182.54	219.84
SEM	14.55	34.71	54.30	40.40	17.00
Left DRG (NT-3)		266.90	65.59	329.10	185.18
		217.90	406.00	270.75	383.65
		106.09	348.38	93.40	403.62
		68.16	241.68	187.59	253.57
		271.63	304.97	175.41	245.98
Mean		186.13	273.32	211.25	294.40
SEM		41.93	58.48	40.72	42.33

Appendix 17: $p75^{NTR}$ mRNA, examined by real-time PCR (copies/10000 copies GAPDH)

Group	Unoperated	1 day	1 week	2 weeks	4 weeks
Right DRG (vehicle)	181.20	7.67	8.96	7.25	144.32
	351.57	11.95	12.14	6.80	229.96
	45.35	18.45	13.16	13.31	214.70
	96.22	23.97	16.69	194.88	59.98
	235.70	19.98	10.42	78.79	219.96
Mean	182.00	16.40	12.28	60.20	173.78
SEM	53.70	2.92	1.32	36.29	32.23
Right DRG (NT-3)		6.45	29.62	88.64	4.39
		6.48	8.83	108.51	208.12
		6.87	22.95	40.06	446.66
		24.58	30.45	99.40	80.04
		15.58	27.97	58.12	235.61
Mean		11.99	23.96	78.94	194.96
SEM		3.60	4.00	12.91	75.73
Left DRG (vehicle)	182.01	7.49	3.51	103.45	180.07
	223.54	3.24	3.20	273.42	54.03
	138.14	13.86	10.00	96.87	388.01
	46.50	8.34	8.97	10.38	116.97
	160.46	4.58	5.53	150.25	3.38
Mean	150.13	7.50	6.24	126.87	148.49
SEM	29.49	1.84	1.39	43.04	66.84
Left DRG (NT-3)		461.72	442.51	332.77	296.64
		288.27	783.95	152.47	420.63
		2.47	473.71	483.79	900.00
		344.57	521.67	816.58	499.24
		238.82	377.62	439.62	368.94
Mean		267.17	519.89	445.04	497.09
SEM		75.88	70.03	109.03	106.02

Appendix 18: Genes, grouped according to functional activity, whose expression was increased following sciatic nerve axotomy, compared to unoperated animal.

Group	Gene name	Accession number (GenBank)	Fold change (Mean ± SD)
Signal transduction and regulation gene expression			
	Mothers against dpp 1 homolog (Mad1)	U66478	4.50 ± 2.15
	Interferon-gamma inducing factor isoform alpha precursor (IGIF)	U77777	3.44 ± 1.01
	VGF	M74223	3.44 ± 1.41
	Immediate-early serum-responsive JE gene (MCP-1)	X17053	2.82 ± 0.29
	Cytosolic retinol-binding protein (CRBP)	M19257	1.88 ± 0.67
	Transferrin	D38380	1.70 ± 0.26
	Guanine nucleotide binding protein beta 1 subunit	AF022083	1.57 ± 0.68
	Microtubule associated protein 2c (MAP2c)	X17682	1.56 ± 0.16
	Janus protein tyrosine kinase 1, JAK1	AJ000556	1.55 ± 0.30
	G protein beta1 subunit (rGb1)	U88324	1.55 ± 0.46
	Ras GTPase-activating protein	D30734	1.52 ± 0.30
Pain-related genes			
	Neuropeptide Y	M15880	54.53 ± 18.61
	Galanin	J03624	25.79 ± 7.51
	Corticotropin releasing factor (CRF)	M54987	1.92 ± 0.74
	P2X2-3 receptor (P2X2)	AF020756	1.51 ± 0.31
Receptor and			

membrane protein			
	Peripheral-type benzodiazepine receptor (PKBS)	J05122	4.31 ± 0.67
	MRC OX-45 surface antigen	X13016	2.19 ± 1.98
	Neuronal high affinity glutamate transporter	D63772	2.06 ± 0.86
	Glutamate/aspartat e transporter protein	X63744	1.95 ± 0.29
	GABA-A receptor alpha-5 subunit	X51992	1.95 ± 0.62
Growth-associated protein and growth factors			
	GAP-43 gene	L21192	2.40 ± 0.56
	Basic fibroblast growth factor	X07285	2.00 ± 0.55
	Neurotrophin-3	M34643	1.97 ± 0.51
	Brain-derived neurotrophic factor (BDNF)	S76758	1.65 ± 0.42
	Insulin-like growth factor binding protein (IGFBP-6)	M69055	1.63 ± 0.48
	Insulin-like growth factor II	X17012	1.57 ± 0.68
Ion transport			
	Dihydropyridine-sesitive L-type calcium channel alpha-2 subunit (CCHL2A) gene	M86621	5.19 ± 1.98
	Brain sodium channel III	Y00766	1.98 ± 0.73
	Neuronatin alpha	U08290	1.93 ± 0.44
	Neuron specific calcium-binding protein hippocalcin	D12573	1.87 ± 1.23
Secreted and extracellular molecules			

	Synaptic vesicle protein (SV2)	L05435	2.28 ± 1.14
	Synaptotagmin IV homolog	U14398	1.73 ± 0.47
	Syntaxin B	M95735	1.53 ± 0.47
	Synaptotagmin associated 35kDa protein	D12519	1.52 ± 0.28
Cytoskeleton			
	Glial fibrillary acidic protein alpha (GFAP) gene	AF028784	3.45 ±1.41
	Microtubule-associated protein 1B	U52950	2.94 ± 2.83
	Gene encoding cytoplasmic beta-actin		1.95 ± 1.20
	RB109 (brain specific protein)	D26154	1.73 ± 0.56
	Mu-calpain large subunit (cls1)	U53858	1.45 ± 0.69
Apoptosis			
	Calmodulin-dependent protein kinase II-delta	J05072	2.27 ± 1.13
Others			
	Heat shock protein (Hsp27)	M86389	2.97 ± 0.65
	Monoamine oxidase A	S45812	2.68 ± 0.21
	Manganese-containing superoxide dismutase (MnSoD)	Y00497	2.43 ± 0.78
	A rat novel protein which is expressed with nerve injury	E12625	1.70 ± 0.52
	Tissue-type plasminogen activator (t-PA)	M23697	1.65 ± 0.63
	Neural adhesion molecule F3	D38492	1.55 ± 0.47
EST			
	Rc_AI101255		2.63 ± 0.96

	EST210544		
	rc_AI105223 EST214512		2.45 ± 0.76
	rc_AI237836 EST234398		2.17 ± 0.98
	rc_AA900476 EST		2.14 ± 0.56
	rc_AA894330 EST198133		2.12 ± 0.86
	rc_AA892559 EST196362		1.98 ± 1.24
	rc_AA925248 EST		1.97 ± 0.93
	rc_AI008639 EST203090		1.82 ± 0.76
	rc_AI176658 EST220250		1.76 ± 0.41
	rc_AI232379 EST229067		1.75 ± 0.52
	rc_AA892801 EST196604		1.70 ± 0.47
	rc_AI044610 EST		1.68 ± 0.94
	rc_AI171319 EST217274		1.61 ± 0.49
	rc_AA817892 EST		1.59 ± 1.04
	rc_AI009268 EST203719		1.58 ± 0.05
	rc_AA998683 EST		1.57 ± 0.37
	rc_AI145494 EST		1.55 ± 0.87
	rc_AI145494 EST		1.54 ± 0.65
	rc_AA925495 EST		1.54 ± 0.18
	rc_AA892814 EST196617		1.54 ± 0.83
	rc_AI029920 EST		1.54 ± 0.46
	rc_AI009405 EST203856		1.51 ± 0.66
	rc_AI072770 EST		1.51 ± 0.51

Appendix 19: Genes, grouped according to functional activity, whose expression was decreased following sciatic nerve axotomy, compared to unoperated animal.

Group	Gene name	Accession number (GenBank)	Fold change (Mean ± SD)
Signal transduction and regulation gene expression			
	Noggin	U31203	-1.89 ± 0.26
	GTP-binding protein (G-alpha-0)	M17526	-1.78 ± 0.23
	Phosphodiesterase I	D28560	-1.71 ± 0.35
	Presenilin-2	X99267	-1.54 ± 0.15
	Ca2+/calmodulin-dependent protein kinase IV kinase isoform	S83194	-1.51 ± 0.31
Pain-related genes			
	Beta-tachykinin	M15191	-2.03 ± 0.73
	Beta-type calcitonin gene-related peptide (CGRP)	M11596	-1.80 ± 0.32
	RET ligand 2 (RETL2)(GDNFR)	U97143	-1.79 ± 0.51
	substance P precursor	X56306	-1.60 ± 0.17
	Somatostatin-14	K02248	-1.60 ± 0.39
	Nicotinic acetylcholine receptor alpha 3 subunit	L31621	-1.57 ± 0.31
	Neuronal nicotinic acetylcholine receptor subunit beta4	U42976	-1.54 ± 0.13
	Somatostatin	M25890	-1.52 ± 0.23
Receptor and membrane protein			
	Glutamate receptor subunit 5-2 (GluR5-2)	M83561	-2.13 ± 0.45
	Neural membrane protein 35	AF044201	-1.86 ± 0.62

	ET-B endothelin receptor	X57764	-1.83 ± 0.79
	Fast nerve growth factor receptor ($p75^{NTR}$)	X05137	-1.69 ± 0.12
	Transferrin receptor	M58040	-1.65 ± 0.26
	Meotropic glutamate receptor (GLUR4)	M90518	-1.63 ± 0.31
	GABA-B receptor gb2	Af058795	-1.60 ± 0.34
Ion transport			
	Potassium channel, alpha subunit (Kv9.3)	Y17607	-3.81 ± 0.78
	CLC-5 chloride channel	Z56277	-2.62 ± 0.92
	Voltage-gated Na channel alpha subunit NaN	AF059030	-2.48 ± 0.64
	Potassium channel (Kv2.2; CDRK)	M77482	-2.16 ± 0.38
	Voltage-gated sodium channel (SNS)	X92184	-2.00 ± 0.89
	Na,K-ATPase alpha-1 subunit	M28647	-1.91 ± 0.31
	5HT3 receptor subunit	U01227	-1.85 ± 0.32
	5HT3 receptor	U59672	-1.85 ± 0.66
	Sodium channel I	M22253	-1.77 ± 0.46
	Potassium channel, alpha subunit (Kv9.1)	Y17606	-1.71 ± 0.35
	Na+,K+-ATPase beta-3 subunit	D84450	-1.55 ± 0.09
	Putative chloride channel	Z36944	-1.55 ± 0.26
	Putative potassium channel TWIK	AF022819	-1.54 ± 0.29
	S100 alpha	S68809	-1.52 ± 0.23
	Potassium channel protein (3145 bp)	X62840	-1.51 ± 0.12
Secreted and			

extracellular molecules			
	SNAP-25A synaptosomal-associated protein 25	AB003991	-1.73 ± 0.72
	Ca2+/calmodulin-dependent protein kinase II isoform gamma-b (CAMK2)	S71570	-1.61 ± 0.14
	Rat ras-related mRNA rab3	X06889	-1.55 ± 0.41
Cytoskeleton			
	Neurofilament protein- light chain (NF-L) mRNA	M25638	-2.12 ± 0.15
	Microtubule-associated protein 1A (MAP1A)	M83196	-1.55 ± 0.29
	Heavy neurofilament polypeptide NF-H C-terminus	X13804	-1.52 ± 0.48
Others			
	Neurodegeneration associated protein 1	D32249	-1.53 ± 0.33
EST			
	Rc_AI230211 EST226906s		-2.53 ± 0.60
	Rc_AI072060 EST		-2.38 ± 0.61
	Rc_AI228113 EST224808		-2.10 ± 0.76
	Rc_AA924772 EST		-1.81 ± 0.36
	Rc_AI013765 EST208440		-1.74 ± 0.54
	Rc_AI136564 EST		-1.54 ± 0.24
	Rc_AI059948 EST		-1.52 ± 0.26

Appendix 20: Genes, grouped according to functional activity, whose expression was increased following sciatic nerve axotomy and NT-3 treatment compared to vehicle treatment.

Group	Gene name	Accession number (GenBank)	Fold change (Mean ± SD)
Signal transduction and regulation gene expression			
	Phosphoinositide 3-kinase regulatory subunit p85alpha	U50412	2.99 ± 0.44
	Neuron-specific cortexin	L15011	1.97 ± 0.74
	CAMP response element binding protein (CREB)	X14788	1.67 ± 0.32
	Mothers against dpp 1 homolog (Mad1)	U66478	1.52 ± 0.47
	GTP-binding protein (G-alpha-0)	M17526	1.50 ± 0.40
Pain-related genes			
	Major hippocampal somatostatin receptor (SSTR4)	U04738	1.69 ± 0.41
	Calcitonin receptor-like receptor (CRLR)	L27487	1.67 ± 0.32
	Muscarinic receptor m2	AB017655	1.67 ± 0.49
Receptor and membrane protein			
	AMPA-selective glutamate receptor-A	S56679	1.92 ± 1.23
	ET-B endothelin receptor	X57764	1.50 ± 0.36
	Chemokine CX3C	AF030358	1.50 ± 0.26
Growth-associated protein and growth factors			
	Fibroblast growth factor (FGF-18)	AB004638	1.72 ± 0.90

Ion transport			
	Potassium channel	M77482	1.73 ± 0.30
	Calcium channel alpha-1S subunit (ROB1)	U31816	1.51 ± 0.78
Secreted and extracellular molecules			
	Syntaxin 4	L20821	1.71 ± 1.03
Cytoskeleton			
	Cytoplasmic beta-actin		1.67 ± 0.34
	Glial fibrillary acidic protein alpha (GFAP)	AF028784	1.54 ± 0.40
Others			
	Phosphoneuroprotein 14	D17764	1.57 ± 0.29
	Calnexin	L18889	1.55 ± 0.25
	Olfactory receptor-like protein (SCR D-8)	AF034896	1.55 ± 0.51
EST			
	Rc_AI146214 EST		1.94 ± 0.74
	Rc_AA893870 EST197673		1.88 ± 0.87
	Rc_AI103671 EST212960		1.72 ± 0.80
	Rc_AI030286 EST		1.70 ± 0.54
	Rc_AI008865 EST203316		1.61 ± 0.49
	Rc_AI029920 EST		1.56 ± 0.29
	Rc_AI228113 EST224808		1.53 ± 0.83
	Rc_AA900476 EST		1.51 ± 0.24
	Rc_AI232379 EST229067		1.50 ± 0.26

Appendix 21: Genes, grouped according to functional activity, whose expression was decreased following sciatic nerve axotomy and NT-3 treatment compared to vehicle treatment.

Group	Gene name	Accession number (GenBank)	Fold change (Mean ± SD)
Signal transduction and regulation gene expression			
	Rat immediate-early serum-responsive JE gene (MCP-1)	X17053	-1.58 ± 0.16
	G protein beta1 subunit (rGb1)	U88324	-1.58 ± 0.12
Receptor and membrane protein			
	Beta-arrestin 2	M91590	-1.50 ± 0.26
Ion transport			
	Sodium/potassium ATPase alpha-1 subunit truncated isoform	M74494	-1.95 ± 0.31
	Voltage-dependent sodium channel alpha subunit [SS1-SS2 segment, transmembrane segments IVS3-IVS6]	S75991	-1.56 ± 0.57
Secreted and extracellular molecules			
	SNAP-25B	AB003992	-1.68 ± 0.58
	Synaptojanin	AJ006855	-1.59 ± 0.40
	Rat ras-related mRNA rab3	X06889	-1.58 ± 0.46
	Synapsin Ia	M27812	-1.54 ± 0.37
	SNAP-25A	AB003991	-1.53 ± 0.53
Cytoskeleton			
	Microtubule-associated protein 1B	U52950	-2.53 ± 0.83
Others			

	Tissue-type plasminogen activator (t-PA)	M23697	-1.71 ± 0.50
	A rat novel protein which is expressed with nerve injury	E12625	-1.51 ± 0.44
EST			
	Rc_AA892801 EST196604		-1.92 ± 1.01
	Rc_AA943304 EST198803		-1.50 ± 0.16

Appendix 22: Real-time PCR confirmation of microarray data.

	Unoperated	2-week axotomy + vehicle	2-week axotomy + NT-3
Neurofilament-L	8599.21744	522.1465077	217.2373692
	9162.292448	1954.931973	2481.935924
	6467.584369	3382.137628	3506.772908
	1426.287051	4670.017741	2885.790885
		4130.503145	3837.445573
Mean	6413.845327	2931.947399	2585.836532
S.E.M.	1760.881367	755.7048071	637.4040691
Neuropeptide Y	8.747903857	5438.67121	4524.615583
	81.46759507	7827.380952	7791.411043
	35.53499598	16442.16691	9290.836653
	46.81172291	2933.175636	9461.662198
	38.33658346	5463.050314	3926.70537
Mean	42.17976026	7620.889005	6999.04617
S.E.M.	11.7081189	2337.205401	1172.746949
5HT-3R	56.7803242	58.13458262	31.52345264
	185.8596679	28.7414966	66.13496933
	107.4818986	57.86237189	104.1434263
	178.9076377	79.24305145	47.20643432
	114.5865835	29.5990566	20.86357039
Mean	128.7232224	50.71611183	53.97437059
S.E.M.	24.09343058	9.614319429	14.68160087
$P75^{NTR}$	181.20	7.25	88.64
	351.57	6.80	108.51
	45.35	13.31	40.06
	96.22	194.88	99.40
	235.70	78.79	58.12
Mean	182.00	60.20	78.94
S.E.M.	53.70	36.29	12.91
Heat shock protein 70	21.92286193	40.58773424	25.34177542
	41.31203931	44.43027211	25.28289025
	35.67176187	91.06881406	55.70517928
	47.06927176	24.95564755	68.86863271
	25.85803432	23.66352201	24.81132075
Mean	34.36679384	44.94119799	40.00195968
S.E.M.	4.682642156	12.24567756	9.333283012
Peripheral-type benzodiazepine receptor	41.74399106	280.9625213	241.2197958
	176.9683985	355.4421769	304.2944785
	96.05792438	445.9736457	245.2589641
	54.55150977	168.361916	417.2654155
	76.09204368	268.0031447	153.0478955
Mean	89.08277348	303.7486809	272.2173099

S.E.M.	23.84812715	46.38247873	43.56423149
Glutamate receptor subnit 5-2	32.2861934	21.61839864	4.155059133
	31.96572041	16.59651361	3.646216769
	7.682220434	2.045095168	7.688446215
	26.67184725	5.441159077	22.69168901
	18.66029641	25.38522013	12.95355588
Mean	23.45325558	14.21727732	10.2269934
S.E.M.	4.650484854	4.529583746	3.531444522

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